CHARACTERIZATION OF A SMALL DOMAIN IN THE I3 LOOP OF THE HUMAN MUSCARINIC M_1 RECEPTOR

By

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Bachelor of Science

Oklahoma State University

Stillwater, Oklahoma

1993

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
In partial fulfillment of
The requirements for
The Degree of
MASTER OF SCIENCE
July, 2006

CHARACTERIZATION OF A SMALL DOMAIN IN THE I3 LOOP OF THE HUMAN MUSCARINIC M_1 RECEPTOR

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ACKNOWLEDGMENTS

I would like to thank Dr. Gregory Sawyer for the opportunity to finish my program in his laboratory and for the pharmacology knowledge he so eloquently shared. Dr. Sawyer is a superb teacher with a great passion for his work. I would also like to thank my other committee members, Dr. David Wallace and Dr. Earl Blewett, for their time and advice.

On a personal note, I would like to acknowledge my dear friend Sarah Myer for her companionship through the years in the lab. Her support and encouragement thru the trying times has become invaluable.

Acknowledgments would not be complete without thanking my two biggest fans, my husband James and son Cody. They have stood by me in the rough times and celebrated with me in the successes even though they never really understood what I was talking about.

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LIST OF ABBREVIATIONS

aa	**amino acid**
DAG	**diacylglygerol**
FBS	**fetal bovine serum**
GPCR	**G protein-coupled receptor**
GRK	**G protein-coupled receptor kinase**
hM_1	**human muscarinic receptor 1**
i3	**intracellular loop 3**
IP_3	**inositol-1,4,5-triphosphate**
mAChR	**muscarinic acetylcholine receptor**
MAP	**mitogen-activated protein**
nAChR	**nicotinic acetylcholine receptor**
[^{3}H]NMS	**[^{3}H]*N*-methylscopolamine**
PBS	**phosphate buffered saline**
PCA	**perchloric acid**
PIP_2	**phosphoinositol-4,5-diiphosphate**
PKA	**protein kinase A**
PKC	**protein kinase C**
TM	**transmembrane**
WT	**wild-type**

Chapter I

BACKGROUND AND INTRODUCTION

Muscarinic acetylcholine receptors (mAChRs) are cell surface receptors which mediate responses to the endogenous neurotransmitter acetylcholine at parasympathetically innervated effector tissues (Wess *et al.*, 1996). The mAChRs are expressed in nearly all parts of the central nervous system. In the central nervous system the mAChR subtype 1 (M_1) has been discovered to play a role in processes of cognition (Messer *et al.*, 1990), learning, control of movement, and the initiation of seizures (Hamilton *et al.*, 2001). Complete understanding of the multiple mechanisms of M_1 receptor function and regulation would allow progression in drug design and development for clinical treatment. The goal of this research is to understand more thoroughly the role of individual amino acid residues in a small domain in the human mAChR subtype 1 (hM_1) intracellular loop 3 (i3) on receptor internalization.

1.1 History of Muscarinic Receptors

Preliminary studies as early as 1869 identified a substance, which was called muscarine, from the poisonous mushroom (*Amanita muscaria*) that would slow, and at high concentrations arrest, the beat of a frog heart (Brown, 1989).

In 1877, Schmiedeberg and Harnack found that when choline was treated with fuming nitric acid, a substance was formed that appeared to have the same pharmacological actions as muscarine (see review, Brown, 1989). Further investigation by Henry Hallet Dale in 1914 found that the pharmacology of the two substances, muscarine and the Schmiedeberg substance, were different and that the choline derived substance, later identified as acetylcholine, also had considerable nicotinic activity (see review, Brown, 1989). This discovery allowed for the classification of the actions of acetylcholine to be divided into nicotinic and muscarinic actions. Muscarinic (mAChR) and nicotinic (nAChR) cell surface receptors are both activated by the same neurotransmitter acetylcholine but are members of two quite different receptor gene superfamilies. Muscarinic receptors are selectively activated by muscarine and blocked by atropine, an alkaloid derived from the deadly nightshade (*Atropa belladonna*); nicotinic receptors are activated by nicotine and blocked by *d*-tubocurarine (Hulme *et al.*, 1990).

1.2 Classification of Muscarinic Receptors

As shown in Figure 1, muscarinic receptors are class 1 rhodopsin-like G-coupled receptors (GPCR) with an extracellular N-terminus, seven transmembrane (TM) domains, three extracellular loops, three intracellular loops and an intracellular C-terminus tail (Hulme *et al.*, 1990). The transmembrane regions have the highest homology between the different subtypes of muscarinic

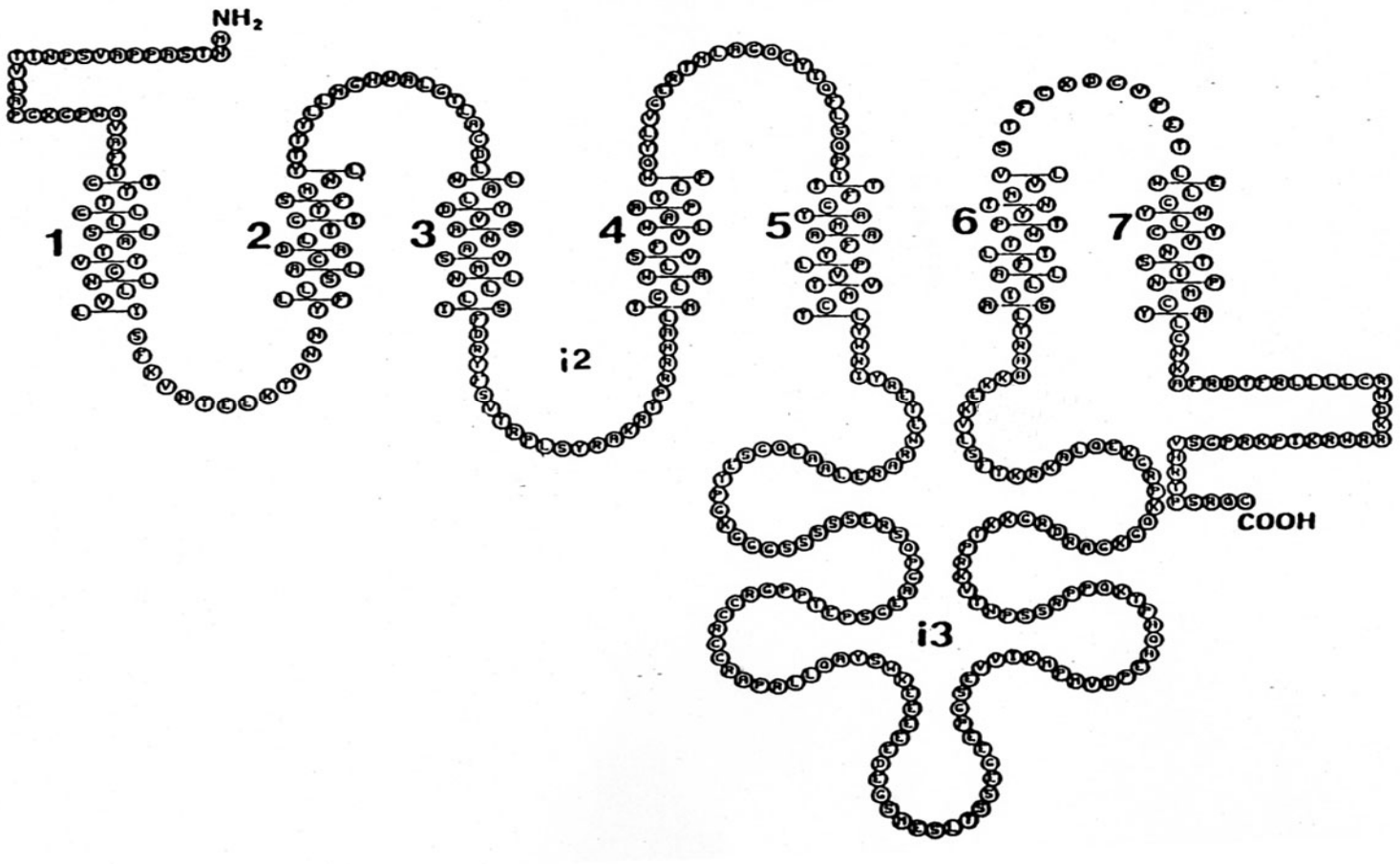

Figure 1. Diagram of the protein sequence of human mAChR 1 (Shockley, 1991)

receptors and other members of the G-protein family (Hulme *et al.*, 1990). Ligand binding to the TM core of the receptor changes the conformation of the receptor activating G-proteins, setting off a course of actions attributed to all GPCRs (Dohlman *et al.*, 1991; Savarese and Fraser, 1992; Strader *et al.*, 1994). Binding of the ligand is predicted to occur specifically in a pocket formed by the transmembrane domains and involves several conserved amino acid residues (Wess, 1993).

1.2.1 Subtypes of Muscarinic Receptors

Historically, receptor classification has been approached by the discovery of receptor selective antagonists and agonists. Receptors are defined by the agonist(s) and antagonist(s) that bind but in the case of muscarinic receptors this approach suggested the existence of subtypes. For example, the antagonist pirenzepine, which has a higher affinity for muscarinic receptors found in neuronal tissue over receptors found in cardiac and smooth muscle, gave the first indication of muscarinic receptor subtypes (Hammer *et al.*, 1980). All subtypes of muscarinic receptors bind the agonist acetylcholine and the antagonist atropine. Unfortunately, no exclusively selective agonist has been described for muscarinic receptors. Complicating further classification, selective antagonists show relatively low selectivity (i.e., 10 to 20 fold lower affinity) between the subtypes (Brown, 1989).

Utilizing pharmalogical binding studies, and advancements in molecular biology, five subtypes of muscarinic receptors have been identified. These five

subtypes of human mAChRs (hM_1 – hM_5), encoded by separate genes, have been cloned (Bonner *et al.*, 1987; Caulfield and Birdsall, 1998; Kubo *et al.*, 1986; Peralta *et al.*, 1987). Each mAChR subtype is encoded by a single exon and expressed as a single polypeptide *in vivo* (Matsui *et al.*, 1999).

Comparing the sequences of the M_1 – M_5 receptors, the major amino acid differences are located in the extracellular amino terminus, the cytoplasmic carboxy terminus, and the third intracellular loop (i3). The greatest difference in sequences between the subtypes are in the i3 loop but there is more homology between M_1, M_3, and M_5 receptors, than M_2 and M_4 receptors which are more homologous to each other. The homology of the i3 loop between M_1, M_3, and M_5 receptors, and M_2 and M_4 receptors is mainly in the amino- and carboxy-terminal portions, and has been postulated to be involved in the coupling of G-proteins (Wess, 1996).

Muscarinic receptors are expressed in specific cells but tissues can have a mixture of different muscarinic subtypes expressed adding an additional complication in defining subtypes. In general, M_1 is mainly found in the forebrain, especially in the hippocampus and cerebral cortex and ganglia (Caulfield and Birdsall, 1998; Dorje *et al.,* 1991; Felder *et al*., 2001; Levey *et al*., 1991). M_2 is expressed in mammalian myocardium and brainstem (Caulfield and Birdsall, 1998; Ehlert and Tran, 1990). M_2 is also the major muscarinic receptor expressed in smooth muscle (Candell *et al.,* 1990). M_3 is a minor fraction of total receptor population and can be found in smooth muscle, exocrine glands, endocrine glands, and cerebral cortex (Caulfield and Birdsall, 1998). M_4 can be

found in the neostriatum and the forebrain (Caulfield and Birdsall, 1998; Yasuda *et al.,* 1992). M_5 is found at very low levels in the hippocampus, striatum, and brainstem (Eglen and Nahorski, 2000).

1.2.2 M_1 Muscarinic Receptor Subtype

The hM_1 gene resides on chromosome 11 and consists of one large exon containing the entire coding region of the hM_1 receptor (Lucas *et al.*, 2001; Matsui *et al*., 1999). The hM_1 AChR is also called the neuronal muscarinic receptor because it is found predominantly in the cortex and hippocampus (Levey, 1993). The M_1 receptor is involved in many processes such as the initiation of seizures, learning and memory (Hamilton *et al.*, 1998). In the cerebral cortex, gene knockout studies have suggested that the M_1 receptor is responsible for the entire cholinergic component of mitogen-activated protein (MAP) kinase stimulation (Hamilton and Nathanson, 2001), mediating effects on synaptic plasticity and cognition (Rosenblum *et al.*, 2000).

M_1, M_3, and M_5 mAChRs couple preferentially to G proteins of G_q/G_{11} class leading to the breakdown of phosophoinositides and do not inhibit adenylyl cyclase (Offermanns *et al.*, 1994). M_2 and M_4 preferentially couple to G_i and inhibit adenylyl cyclase (Offermanns *et al.*, 1994). As shown in Figure 2, the coupling of M_1 to the G_q/G_{11} G protein leads to the activation of phospholipase C (PLC) which hydrolyzes phosphoinositol-4,5-bisphosphate (PIP_2) to generate the second messengers inositol-1,4,5-triphoshate (IP_3), and diacylglygerol (DAG). IP_3 can be recycled back into PIP_2 or binds to receptors on the endoplasmic

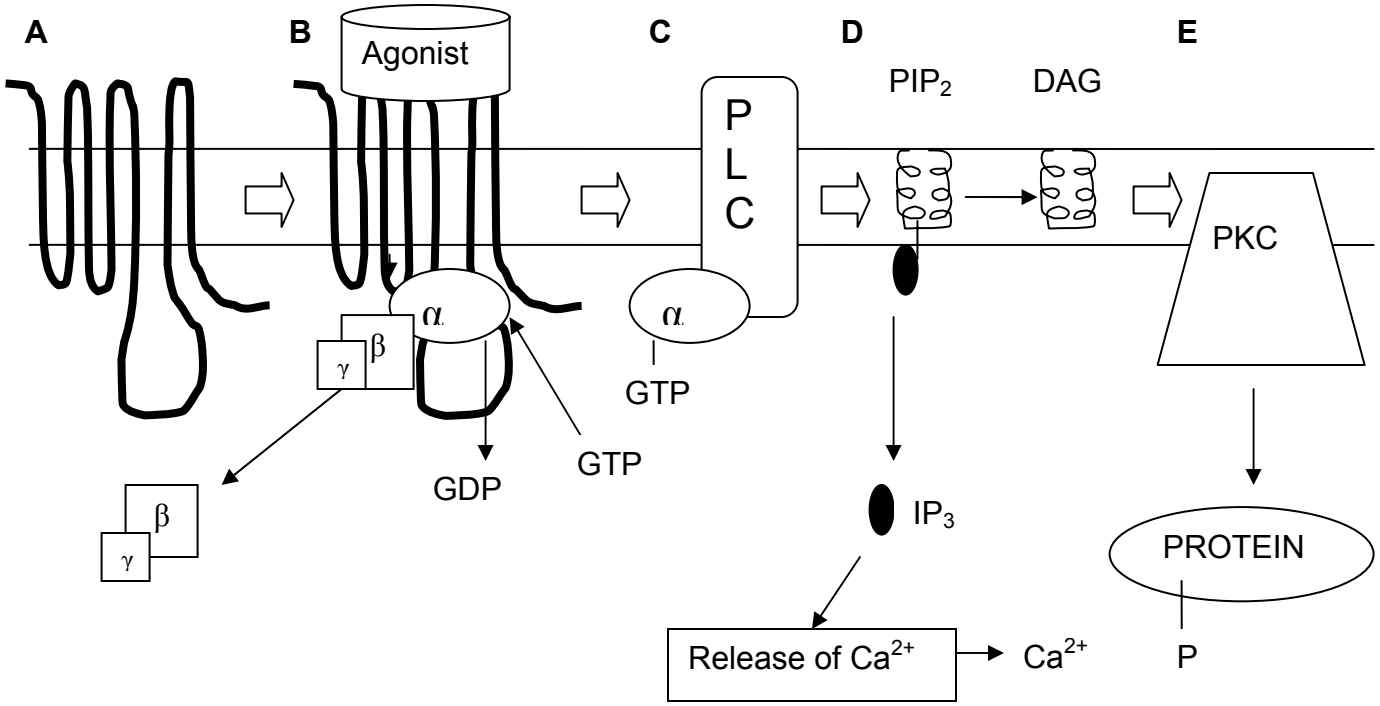

Figure 2. M_1 receptor signaling. A) M_1 receptor. B) Binding of agonist to the M_1 receptor activates the G-protein by initiating the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) on the α subunit. The $G_q/_{11}$ α-subunit dissociates from the $\beta\gamma$ complex. C) The α subunit binds and activates the PLC. D) PLC hydrolyzes PIP_2, which generates IP_3 and DAG. IP_3 binds to receptors on the endoplasmic reticulum to release intracellular Ca^{2+}. E) DAG activates PKC which can phosphorylate proteins.

reticulum to release calcium from intracellular stores (Jones *et al.*, 1990). DAG can be recycled back into PIP_2 through a series of reactions or can activate protein kinase C (PKC). The activated form of PKC can then phosphorylate proteins such as the M_1 receptor.

1.3. Regulation of Expression and Function of AChR

Distribution of G protein coupled receptors on the cell surface is maintained by two processes that transport the receptors throughout the cell. The first process involves newly synthesized receptors from the rough endoplasmic reticulum transported to the Golgi complex, processed and transported to the cell surface by transport vesicles. Endocytosis is the second process and describes receptors moving from the cell surface into internal endosome compartments because of basal rate (slow) endocytosis or agonist-stimulated (fast) endocytosis (Koenig and Edwardson, 1997). The path taken by the majority of receptors after reaching the endosome is to be recycled back to the cell surface, but the receptors can also be directed to lysosomes for degradation (Koenig and Edwardson, 1997).

Prolonged agonist exposure activates a regulatory mechanism that causes desensitization (loss of functional response), quickly ending agonist-induced stimulation. Desensitization can be homologous, whereby only the activated receptors are desensitized, or heterologous, whereby activation of one receptor system may produce signals that feedback on another receptor. Typically desensitization is recognized by the muscarinic receptors losing high

affinity binding, uncoupling from G proteins and internalizing to a ligand inaccessible compartment. Extended (hours) agonist exposure can cause down-regulation of the receptor by either increasing the degradation of existing proteins or by a decrease in the synthesis of new receptors (Wang *et al.,* 1990).

1.3.1 Phosphorylation and Uncoupling from G Proteins

Agonist bound G protein-coupled receptors are phosphorylated by G protein-coupled receptor kinases and cytosolic β-arrestins bind with increased affinity to the receptors and sterically inhibit further coupling of the receptors with G proteins (Krupnick and Benovic, 1998). Phosphorylation of hM_1 receptors normally occurs at serine/threonine residues in the C-terminus tail and/or the i3 loop (Haga et al., 1996).

There are two types of serine/threonine kinases that phosphorylate muscarinic receptors. The first are second messenger kinases that include protein kinase A (PKA) and protein kinase C (PKC). Heterologous desensitization on M_1 receptors can be mediated by PKC and phosphorylation occurs at two to three sites punitively located in the carboxy-terminal part of the i3 loop and the carboxy-terminal tail (Haga et al., 1996). The second type of kinases are the G protein-coupled receptor kinases (GRKs) which phosphorylate receptors that are in an active state (Palczewski and Benovic, 1991). GRKs are a family of seven kinases (GRK 1-7) where GRKs 2,3,4,5, and 6 regulate most GPCRs (Lefkowitz and Shenoy, 2005). Agonist-dependent phosphorylation of hM_1 receptors is thought to occur by GRK2 on four to five sites in a

serine/threonine rich domain located in the central portion of the i3 loop (Haga et al., 1996).

1.3.2 Internalization

Internalization is the loss of receptors on the cell surface without loss of total cellular receptor binding sites. Overall, the mechanism by which agonist stimulation of muscarinic receptors causes internalization is not completely understood, and understanding is complicated by mechanisms differing depending on cell type and subtype.

Internalized M_1 receptor can be recycled to the cell surface or compartmentalized into a lysosome for down-regulation. hM_1 receptors have been shown to internalize into non-coated vesicles in CC137 cells (Raposo *et al.*, 1987), in an arrestin (cytosolic protein) independent but dynamin-dependent pathway in HEK-tsA201 cells (Lee *et al.*, 1998), and in intracellular clathrin-coated vesicles following agonist stimulation in HEK293 cells (Tolbert and Lameh, 1996) and CHO-K1 cells (Shockley *et al.*, 1997). The loss of receptors from the cell surface in response to agonist stimulation differs by cell type in which the receptor is expressed (Koenig and Edwardson, 1997)

The domains correlated with internalization of the M_1 receptor have been shown to be located in the i3 loop. Large deletions of the i3 loop have impaired internalization (Maeda *et al.*, 1990), and various smaller deletions in the i3 loop have been identified to impair or to partially impair internalization (Lameh *et al.*, 1992). The smaller deletion mainly focused on by investigators is a

serine/threonine (286-ESLTSSE-292) rich domain (Moro *et al.*, 1993), which later has been shown to be involved in down-regulation rather that internalization (Shockley *et al.*, 1999).

1.3.3 Down-Regulation

Down-regulation is defined by the loss of total receptor numbers as a consequence of receptor degradation. In contrast to short-term desensitization, which occurs on a time scale of minutes without a decrease in total cellular receptor number, down-regulation occurs over several hours (Taylor *et al.*, 1979). The decreased concentration of receptors after translocation to lysosomes are mechanisms involved in receptor down-regulation (Habecker and Nathanson, 1992; Klein *et al.*, 1979). Since receptor endocytosis precedes receptor down-regulation, specific investigations into down-regulation distinguished from internalization are problematic. hM_1 receptor down-regulation in CHO-K1 cells has been found to be differentially regulated from receptor internalization and mutations that impair down-regulation also affect G-protein coupling (Shockley *et al.*, 1997). In contrast to CHO-K1 cells, HEK293 cells have not shown any ability to down-regulate the M_1 receptor (Moro *et al.*, 1994).

1.4 Significance of Study

The emphasis of this research is to characterize a small domain located in the i3 loop of the hM_1 receptor that has been previously shown to affect agonist-dependent internalization. The mechanism for muscarinic receptor

internalization is not completely understood, and previous research has been based on a larger portion of the i3 loop of the hM_1 receptor. Narrowing down the specific amino acids involved in hM_1 receptor internalization will aide in the future identification of the specific mechanism involved.

1.4.1 Discovery of 8 Amino Acid Domain

Dimerization is a familiar theme in the regulation of signal transduction. Several members of the GPCR category of cell surface receptors, even though generally believed to function as monomers, have been found to function as multimers (Terrillon and Bouvier, 2004). Studies have concluded that dimerization under at least some conditions are common events in muscarinic receptors (Maggio et al., 1993; Park et al., 2001; Wreggett and Wells, 1995; Zeng and Wess, 1999). Human M_2 receptors have been determined to be capable of forming dimmers in S*f*9 cells and retain their dimerization status under a variety of conditions (Park et al., 2001). Deletion analysis of chimeric α_2Adrenergic/M_3 muscarinic receptor chimeras have shown that dimerization can occur in the M_3 receptor (Maggio *et al.*, 1993). Further investigation of dimerization of M_3 receptors implicated that the size of the i3 loop of muscarinic receptor was important in the regulation of receptor dimerization (Maggio et al., 1996).

The implication that the size of the i3 loop in M_3 was important in dimerization led to the investigation of the i3 loop in hM_1 and its capability of interacting (forming dimers) with itself. Previously, our laboratory used the yeast

two-hybrid system to identify an 8 amino acid (aa) domain in the i3 loop of hM_1. Briefly, the yeast two-hybrid system is a molecular genetic tool which utilizes a bait and prey technique which facilitates the study of protein-protein interactions. The bait protein is expressed as a fusion with the GAL 4 DNA binding domain while another protein, prey, is expressed as a fusion to the GAL 4 activation domain. When “bait” and “prey” interact with each other in a yeast reporter strain, the DNA binding domain and activation domain are brought into proximity and activate the transcription of reporter genes. Neither the DNA binding domain nor activation domain can activate a reporter gene alone. In our laboratory, multiple deletions in the hM_1 i3 loop were made as “prey” and expressed with full length i3 loop “bait”. The “prey” deletion constructs activated the reporter genes when expressed with the “bait”, with the exception of one that had a deletion of 8 amino acids (Figure 3).

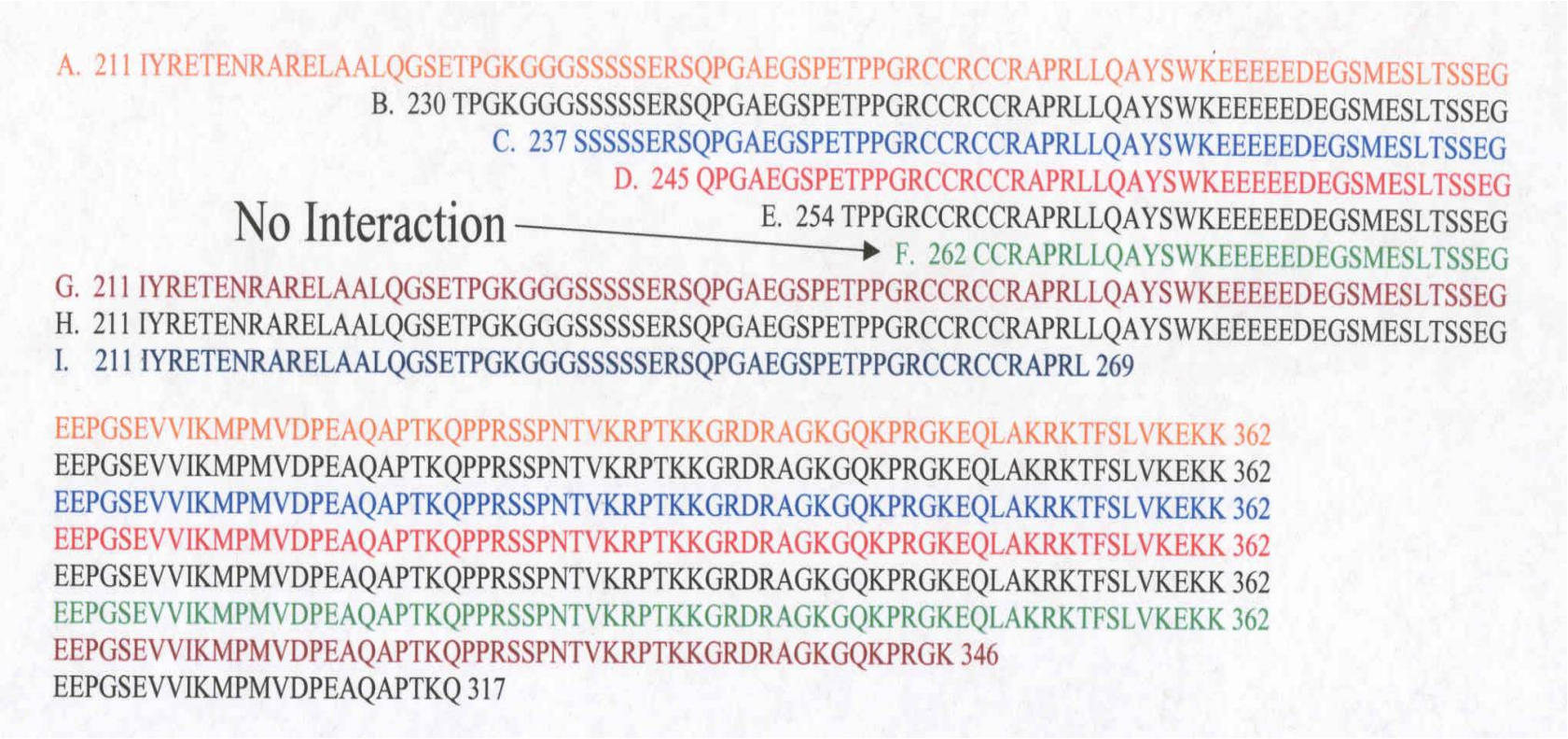

Figure 3. hM_1 i3 loop constructs used in yeast two-hybrid experiments. Multiple deletions of the "prey" i3 loop fusion protein were made (B-I) and expressed with full-length (aa 211-362) "bait" i3 loop fusion proteins (A) in AH109 to identify the domain mediating an interaction between full-length i3 loop fusion proteins. All "prey" i3 loop fusion proteins (B-I) interacted with moderate affinity with the "bait" i3 loop construct except for F, which deleted the 8 amino acids 254-TPPGRCCR-262 (unpublished observations by Ricks, T.K. and Sawyer, G.W.)

A mutant hM_1 receptor was made by mutagenesis, deleting only the 8 amino acids (254-TPPGRCCR-261) from the i3 loop. Pharmacological assays were performed to determine the functional role of this 8 amino acid domain. This mutant hM_1 (hM_1/8 aa del) receptor was compared to wild-type hM_1 (hM_1/WT) receptor, in assays that measure agonist-induced internalization, saturation radioligand binding, phosphoinositide hydrolysis and down-regulation.

It was concluded that the hM_1/8 aa del internalized less extensively than the hM_1-WT receptor when incubated with the agonist carbachol (Figure 4). Although there was a significant difference in internalization of the receptors, the affinity of [^{3}H]NMS binding and the potency of phosphoinositide hydrolysis when elicited to the agonist carbachol, was similar for hM_1/8 aa del mutant and hM_1/WT (unpublished observations). Down-regulation after 24 hr exposure to the agonist carbachol was similar for both the hM_1/8 aa del and hM_1/WT receptor (unpublished observations). Collectively, the data suggests that the 8 amino acid domain in the hM_1 i3 loop plays an important role in agonist-dependent internalization.

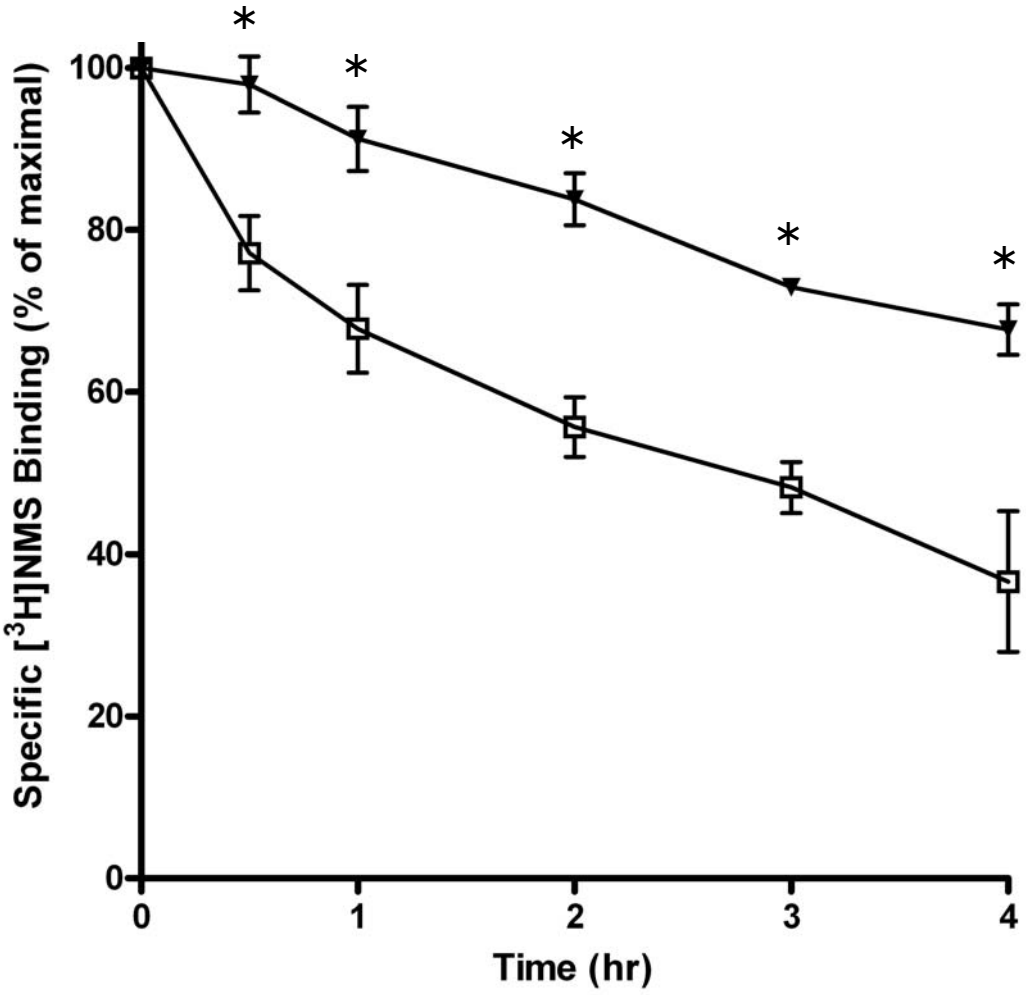

Figure 4. Agonist-dependent internalization of hM_1/WT and hM_1/8 aa del receptors. Mutant hM_1 receptors lacking the 8 amino acid domain 254-TPPGRCCR-261 in the i3 loop, internalized less extensively than the hM_1/WT receptors when incubated with the agonist carbachol. CHO-K1 cells expressing either hM_1/WT (□) or hM_1/8 aa del mutant (▼) were incubated with carbachol (1 mM) for up to 4 hour before conducting binding assays with [^{3}H]NMS. Each data point represents the mean ± S.E.M. of four experiments conducted in triplicate. ✶ Denotes statistically significant differences ($p < 0.05$) between data points determined by student's t-test (unpublished observations by Ricks, T.K. and Sawyer, G.W.)

1.4.2 Experimental Approach to Characterize a Small Domain in the I3 Loop of the hM_1 Receptor

My experimental approach includes three components (Figure 5). The first component of the study includes utilizing a protein motif scan to identify conserved domains within the i3 loop of the hM_1 receptor that could play a role in agonist-dependent internalization. The second component includes producing mutant receptors possessing changes of specific amino acids within each identified conserved domain. The third component includes looking at the agonist-dependent internalization, signaling, and binding of each hM_1 receptor mutant. The signaling and binding assays ensure that the affect(s) of the mutation(s) on internalization are not because of changes in receptor function.

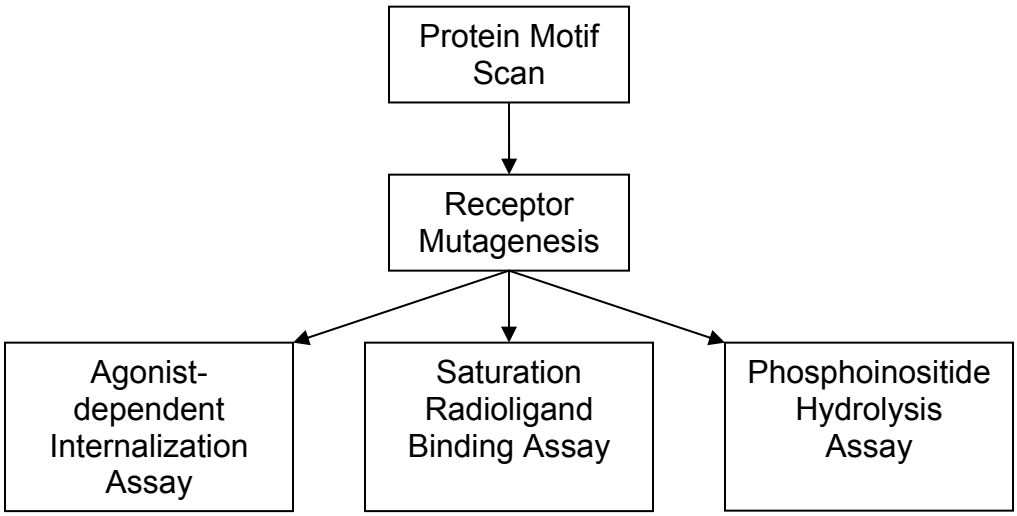

Figure 5. Experimental approach to characterize a small domain in the i3 loop of the hM_1 receptor.

CHAPTER II

RESEARCH METHODS

2.1 pCD-hM$_1$ and Mutagenesis of the hM$_1$ Receptor

Mutant pCD-hM$_1$ vectors were made using pCD-hM$_1$ (generous gift of Dr. Tom Bonner at the NIMH), a modified Okayma-Berg expression vector possessing the hM$_1$ receptor sequence (Bonner *et al*., 1997), and the QuikChange II Site-Directed Mutagenesis Kit. Using the manufacturer's instructions and various mutagenesis primers (see Table I), several different point mutations were introduced into the M$_1$ receptor domain 252-PETPPGRCCRCCRA-265 (see Figure 6). Mutagenesis of the hM$_1$ receptor involved mutant strand synthesis, *Dpn I* digestion of the parental template, and transformation of XL1-Blue *E. coli* with the mutant plasmids. All mutant pCD-hM$_1$ receptor constructs were sequenced at the OSU core facility (Stillwater, OK) to verify the presence of the mutation(s) before use and to ensure that no other mutations were acquired during PCR.

The hM$_1$ receptor sequence of pCD-hM$_1$/T to A has an alanine in position 254 instead of a threonine (Figure 6). Alanine was substituted for cysteine in positions 259 and 260 of the hM$_1$ receptor sequence of pCD-hM$_1$/CC-AA

(Figure 6) and in positions 262 and 263 of the hM_1 receptor sequence of pCD-hM_1/2CC-AA (Figure 6). In positions 259, 260, 262, and 263 of the hM_1 receptor sequence of pCD-hM_1/4CC-AA, and position 263 of pCD-hM_1/2C-A (Figure 6), alanine was substituted for cysteine. The hM_1 receptor sequence of pCD-hM_1 /P-A has alanine in position 252 instead of proline (Figure 6).

WT	252 - P E T P P G R C C R C C R A - 265
T – A	252 - P E **A** P P G R C C R C C R A - 265
CC – AA	252 - P E T P P G R **A A** R C C R A - 265
2CC	252 - P E T P P G R C C R **A A** R A - 265
4CC	252 - P E T P P G R **A A** R **A A** R A - 265
2C - A	252 - P E T P P G R C C R C **A** R A - 265
P - A	252 - **A** E T P P G R C C R C C R A - 265

Figure 6. hM_1/WT and mutant receptor amino acid sequence between residues 252 - 265. Using the QuikChange II site-directed mutagenesis kit and mutagenesis primers (see Table I), point mutations were introduced at various positions in the i3 loop domain 252-PETPPGRCCRCCRA-265 exchanging alanine (bolded) for the WT amino acids.

Table I. Mutagenesis Primers

pCD-hM_1 Mutant	Primers Used
T - A	
Forward	5'GAGGGCTCACCAGAG**G**[1]CTCCTCCAGGCCGCTGC 3'
Reverse	5'GCAGCGGCCTGGAGGAG**C**[1]CTCTGGTGAGCCCTC 3'
CC -AA	
Forward	5'GACTCCTCCAGGCCGC**GCTGC**[1]TCGCTGCTGCCGGGCC 3'
Reverse	5'GGCCCGGCAGCAGCGA**GCAGC**[1]GCGGCCTGGAGGAGTC 3'
2CC	
Forward	5'GGCCGCTGCTGTCGC**GA**C**GAT**[1]CGGGCCCCCAGGCTG 3'
Reverse	5'CAGCCTGGGGGCCCG**ATC**G**TC**[1]GCGACAGCAGCGGCC 3'
4CC	
Forward	5'ACTCCTCCAGGCCGCGACGACCGCGACGA[1]CCGGGCCCCCAGGCTG 3'
Reverse	5'CAGCCTGGGGGCCCGGTCGTCGCGGTCGTC[1]GCGGCCTGGAGGAGT 3'
2C - A	
Forward	5' TGCTGTCGCTGC**GC**[1]CCGGGCCCCCAGG 3'
Reverse	5'CCTGGGGGCCCGG**GC**[1]GCAGCGACAGCA 3'
P - A	
Forward	5'GCTGAGGGCTCA**G**[1]CAGAGACTCCTCCAG 3'
Reverse	5'CTGGAGGAGTCTCTG**C**[1]TGAGCCCTCAGC 3'

[1]Bolded nucleotides represent the changes in the hM_1/WT DNA sequence in the pCD-hM_1 plasmid (Bonner *et al*., 1987).

2.2 Chinese Hamster Ovary (CHO) Cell Line Culture and Transfection

The CHO-K1 cell line was derived as a subclone from the parental CHO cell line initiated from a biopsy of an ovary from an adult Chinese hamster by T.T. Puck in 1957 (Puck *et al.*, 1958). CHO-K1 cells were obtained from ATCC and were stored in the vapor phase of a Locator Jr. cryobiological storage system in aliquots (1 ml) at passage 2 in complete culture medium (F-12K, 10%FBS, 100 units/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate) and 5% DMSO. Frozen cells were thawed at 37°C and revived in complete culture medium. After revival, CHO-K1 cells were maintained in 75 cm^2 flasks in complete culture medium in a humidified incubator at 37°C and 5% CO_2.

For agonist-dependent internalization, phosphoinositide hydrolysis, and saturation ligand binding assays, CHO-K1 cells were trypsinized and seeded in a 24-well plate format at cell density of 1.75 x 10^5 cells per well in 500 μl transfection medium (F-12K, 10% FBS) and incubated in a humidified incubator at 37°C and 5% CO_2 for 24 hours. Cells were transfected using Lipofectamine 2000 according to the manufacturers' protocol. Briefly, 0.8 μg/well plasmid DNA (19.2 μg/plate) was incubated with 50 μl/well Opti-MEM I (1,200 μl/plate) for 5 minutes at room temperature in a 15-ml conical tube. Lipofectamine 2000 (48 μl/plate) was incubated in a separate tube with 50 μl/well Opti-MEM I (1,200 μl/plate) for 5 minutes at room temperature. The plasmid DNA and lipofectamine mixtures were combined into a single tube, mixed gently, and incubated for 20 minutes at room temperature allowing DNA/Lipid complexes to form. Complexes (100 μl/well) were added to each well of the 24-well CHO-K1 plate, rocked gently,

and placed into a humidified incubator set at 37°C in a 5% CO_2 atmosphere. After 6 hours of incubation, the media containing DNA/Lipid complexes was replaced with 500 μl of fresh transfection medium. Cells were incubated an additional 18 hours in a humidified incubator set at 37°C in a 5% CO_2 atmosphere.

2.3 Agonist-Dependent Internalization Assays

To determine the degree of WT and mutant hM_1 receptor internalization in response to agonist, the membrane impermeable muscarinic receptor selective radioligand [^{3}H]NMS was used to determine the amount of plasma membrane expressed receptor before and after incubation with carbachol (a muscarinic receptor selective agonist). Transiently transfected CHO-K1 cells were incubated with 1 mM carbachol in 500 μl of F-12K medium without FBS, for up to 4 hours at 37°C in 5% CO_2 atmosphere. Intact whole cell binding assays were then performed. Cells were rapidly and gently washed twice on ice with 500 μl ice-cold PBS (11.9 mM phosphates, 137 mM NaCl, 2.7 mM KCl). The intact cells were then incubated for 1 hour at 4°C with 1.6 nM [^{3}H]NMS in the absence (total binding) or presence (nonspecific binding) of 0.1 μM of atropine in 500 μl binding buffer (25 mM HEPES, 113 mM NaCl, 6 mM dextrose, 3 mM $CaCl_2$, 3 mM KCl, 2 mM $MgSO_4$, 1 mM NaH_2PO_4, pH 7.4). Following incubation, each well was rapidly, and gently washed twice on ice with ice-cold PBS (1 ml/wash), then incubated with 0.25 N NaOH (500 μl) for 30 minutes at room temperature. 2.5 N HCl (70 μl) was added, and neutralized extracts were then transferred from each

well of the plate into individual 7-ml scintillation vials. Scintiverse (5 ml) was added and the radioactivity was determined using a LS 6500 scintillation counter (Beckman).

Average total protein was determined every experiment for each hM_1 receptor construct characterized. Briefly, 3 wells of a 24-well plate were seeded with CHO-K1 cells and transiently transfected with WT or mutant hM_1 receptor constructs as described in section 2.2. On the day of the experiment, cells were washed two times with 500 μl of mannitol wash buffer (0.29 M mannitol, 0.01 M Tris, 0.5 mM $Ca(NO_3)_2$,pH 7.4), then incubated with 1 N NaOH (1 ml) for 15 minutes at room temperature as described previously (Goldschmidt and Kimelberg, 1989). Cells were then sonicated for 1 minute at room temperature and 1 N HCL (100 μl) was added to neutralize a 100 μl aliquot of cell extract. Bicinchoninic acid (BCA) regent (1 ml) was added as described by Goldschmidt and Kimelberg (1989). The remainder of the assay was conducted according to Pierce (Rockford, IL) manufacture protocol.

2.4 Phosphoinositide Hydrolysis Assays

To determine whether mutant hM_1 receptors could still elicit phosphatidylinositol metabolism when exposed to agonist, CHO-K1 cells transiently expressing the hM_1/WT and mutant receptors were used in phosphoinositide hydrolysis assays. Transiently transfected CHO-K1 cells were incubated with 0.2 μM [^{3}H]myo-inositol in 500 μl F-12K medium. After 18 hours of incubation in a humidified incubator at 37°C and 5% CO_2, cells were washed

twice with 500 μl F-12K medium with a 10 minute incubation at 37°C and 5% CO_2, in between washes. For a final wash, cells were washed with 300 μl F-12K medium containing 10 mM LiCl and incubated for 10 minutes at 37°C and 5% CO_2. The washed cells were then incubated with 300 μl of F-12K medium containing 10 mM LiCl and geometrically spaced concentrations of carbachol (0.5 log unit) for 30 minutes in a humidified incubator at 37°C and 5% CO_2. After incubation, the media was aspirated from each well and cells were incubated for 15 minutes on ice in 200 μl of ice-cold 5% PCA. To each well 365 μl (volume dependent on titration values of individual batch) of 0.525 M KOH in 10 mM Tris-HCl was added and the cells were incubated for an additional 15 minutes on ice. The neutralized PCA extracts were transferred into individual 1.5 ml microcentrifuge tubes and each well was washed with 400 μl of 25 mM Tris-HCl, pH 7.4. Each wash was added to its corresponding microcentrifuge tube and the tubes were centrifuged at 3000 x g for 10 minutes in a microcentrifuge 5415 D (Eppendorf) to pellet any cellular debris. The supernatant was transferred into individual glass test tubes (16 x 100 mm) containing 2 ml of 25 mM Tris-HCl, pH 7.4 and mixed. The solution from each tube was applied to individual 1 ml Dowex AG 1-X8 (formate form, 100-200 mesh) columns. Columns were washed 4 times with 4 ml deionized ultra filtered water and [^{3}H]inositolphosphates were eluted using 2.5 ml of solution containing 1 M ammonium formate and 0.1 M formic acid into 25 ml scintillation vials. To each vial, 20 ml of Scintiverse was added and radioactivity was counted using a LS 6500 scintillation counter.

2.5 Saturation Radioligand Binding Assays

To determine whether the various amino acid substitutions we made affected the affinity of the receptor for ligands or the plasma membrane expression of the receptor, we conducted radioligand binding assays with [^{3}H]NMS. Using this approach, we were able to obtain an estimate of the equilibrium dissociation constant (K_D) for each receptor mutant and determine the maximal number of binding sites (B_{max}) expressed at the plasma membrane. As mentioned in section 2.3, the [^{3}H]NMS is membrane impermeable, so it is a good radioligand for detecting whether there is an affect of the mutations on plasma membrane expression of the receptor.

Transiently transfected CHO-K1 cells were rapidly and gently washed twice on ice with 500 μl per well ice-cold PBS. Intact cells were then incubated at 4°C for 1 hour with geometrically spaced concentrations of [^{3}H]NMS (0.33 log unit) in the absence (total binding) or presence (nonspecific binding) of 0.1 μM of atropine in 500 μl binding buffer. Following incubation, cells from each well were rapidly and gently washed twice on ice with ice-cold PBS (1 ml/wash), then incubated with 0.25 N NaOH (500 μl) for 30 minutes at room temperature. 70 μl of 2.5 N HCl was added and the neutralized extracts were transferred from each well of the plate into individual 7-ml scintillation vials. Scintiverse (5 ml) was added and the radioactivity was determined using a LS 6500 scintillation counter.

2.6 Mathematical Analysis

Specific [^{3}H]NMS binding was calculated for each concentration of [^{3}H]NMS used in radioligand binding assays (see section 2.5) and each time point in agonist-dependent internalization assays (see section 2.3) using equation 1.

$$Specific\ Binding = Total\ Binding - Nonspecific\ Binding \qquad \textbf{eq. 1}$$

Mole specific binding was calculated using equation 2.

$$Mole\ \ Specific\ \ \ Binding\ \ = \frac{Specific\ \ \ Binding\ \ \ (dpm\ )}{(\ X\ )(\ N\ )} \qquad \textbf{eq. 2}$$

In equation 2, X denotes the conversion factor for dpm to Ci (1 Ci = 2.22 x 10^{12} dpm). N denotes the specific activity of [^{3}H]NMS, which in this study was either 81 or 82 Ci/mmol. Femtamole specific binding was calculated using equation 3.

$$Fmol\ Specific\ Binding = Mole\ Specific\ Binding \times \left(1 \times 10^{15}\ \ fmol/mol\right) \qquad \textbf{eq. 3}$$

The percent of maximal specific [^{3}H]NMS binding was calculated for each time point in the carbachol-induced internalization assay by dividing the fmol specific binding at time point 0 hour by the fmol specific binding at time point 0.5, 1, 2, 3.

or 4 hour and multiplying by 100. Non linear analysis for PI assays and saturation binding assays was calculated using equation 4.

$$y = \min + \frac{\max - \min}{1 + \left(10^{\log K_D \text{ or } EC_{50}} / 10^{\log\left([^3H]NMS\right)}\right)^n} \qquad \textbf{eq. 4}$$

In this equation, min denotes minimum specific [^{3}H]NMS binding, max denotes B_{max} in binding assays or E_{max} in PI assays, and n denotes the hill coefficient (Bowen and Jerman, 1995). Mole specific binding per mg protein was calculated by dividing the mole specific [^{3}H]NMS binding by the mean mg protein determined in the BCA assay described in section 2.3.

2.6.1 Statistical Analysis

Data from the agonist-dependent internalization assay was analyzed by two-way ANOVA, to test for overall main effect or effect of mutation, time, or relationship between time and mutation, followed by a Bonferroni analysis corrected for multiple comparisons. A one-way ANOVA, to test for effect of mutation, with Bonferroni post-test was used to analyze the data from the saturation radioligand binding assay, and the phosphoinositide hydrolysis assay. $p < 0.05$ was considered statistically significant for all tests. ANOVA analysis was performed using GraphPad-Prism software, version 4.03 (GraphPad, San Diego, CA).

2.7 Materials

All reagents and labware were obtained from Fisher Scientific (USA) except for the following: The QuikChange Mutagenesis Kit was purchased from Stratagene (La Jolla, CA). CHO-K1 cells, FBS, and F-12 medium were purchased from ATCC (Manassas, VA). Lipofectamine 2000, penicillin G sodium/Streptomycin sulfate, primers for mutagenesis, and Opti-Mem 1 were obtained from Invitrogen (Carlsbad, CA). DMSO, carbachol, atropine, HEPES, LiCl, PCA, ammonium formate, formic acid, were purchased from Sigma (St. Louis, MO). Radioactive reagents, [^{3}H]NMS and [^{3}H]*myo*-inositol, were purchased from PerkinElmer Life and Analytical Science (Boston, MA). BCA reagent kit was manufactured by Pierce (Rockford, Il.). Microcentrifuge 5415D was manufactured by Eppendorf (Westbury, NY). Dowex AG 1-X8 was purchased from Bio-Rad (Hercules, CA). The LS 6500 scintillation counter was manufactured by Beckman Coulter (Fullerton, CA).

CHAPTER III

RESULTS

3.1 General Overview of Results

Previously in our laboratory, a small 8 amino acid deletion in the third intracellular loop was found to decrease the extent of agonist-induced internalization of hM_1 receptors (unpublished observation) and is shown in Figure 3. Characterization of this small 8 amino acid domain prompted investigating the amino acids within and surrounding the domain to identify all amino acids that may play a role in agonist-dependent internalization. Once the amino acid(s) that potentially play a functional role in the domain were identified, mutants were then made by replacing the existing amino acid(s) with alanine to determine whether the amino acids were involved in the previously seen affect of deleting aa 254-261 on agonist-induced internalization. The mutant hM_1 receptors were then compared to hM_1/WT receptors in relevant assays (Figure 5). The agonist-dependent internalization assay was used to identify the amino acid(s) that play a role in internalization. Saturation radioligand binding assays were used to determine the affinity of the ligand for the receptors. Phosphoinositide hydrolysis assays were used to determine whether or not the mutant receptor elicits phosphoinositide hydrolysis to agonist.

3.2 Identification of Protein Sequence Motifs Within or Overlapping with a Small Domain in the I3 Loop of hM_1 Receptors

Utilizing a web-based protein motif scan program (http://scansite.mit.edu), the 8 amino acid domain found previously and surrounding amino acids were searched for consensus sequences of known biological function.

The protein motif scan, which identifies signatures of protein families, retrieves all of the respective family members from the sequence and allows classification of an unknown sequence based on the homology of known proteins (Bork and Gibson, 1996; Bork and Koonin, 1996). If the sequence motif matches 100%, the score is 0.00 and as the sequence diverges from the match, the score increases. The percentile value given for a motif scan is calculated from the vertebrate section of the Swiss-Prot reference database (Obenauer *et al.*, 2003). A high stringency scan identifies the sequence motifs that match in the best 0.2% of all sites available where the medium stringency scan highlights the sequence motifs that match in the best 1% of all sites available. Two potential domains of known function were found in the i3 loop of hM_1 receptors using this approach. These domains were in close proximity to the 8 amino acid domain previously identified.

The first domain of known function, found in a medium stringency motif scan, was a putative amphiphysin SH3 binding domain (245-QPGAEGS**P**ETPPGRC-259), with a score of 0.5784 in the 0.913 percentile. The second domain of known function, found with a high stringency motif scan, was a putative proline-dependent serine/threonine kinase (Pro_ST_Kin) Cdc 2 kinase

phosphorylation site (247-GAEGSPE**T**PPGRCCR-261) with a score of 0.4276 and 0.147 percentile.

The 8 amino acid domain and adjacent amino acids contain several cysteine residues (254-TPPGRCCR-262 + 263-CCR-265). Disulfide bonds can be formed between the side chains of adjacent cysteines (Carugo *et al.*, 2003), raising the possibility that vicinal (adjacent) disulfide linkages may play a part in the affect on internalization seen previously. Extracellular cysteines forming disulfide bridges have been shown to participate in the formation of disulfide-linked M_3 receptor dimers (Zeng and Wess, 2000), but studies on intracellular adjacent cysteines have not been addressed. Also, vicinal disulfide bonds can play a structural role in the protein backbone conformation (Carugo *et al.*, 2003). Conformational changes, because of expression of mutant cysteine to alanine hM_1 receptors, could possibly alter an unknown mechanism in agonist-dependent internalization.

The hM_1 receptor was mutagenized based on the information from the protein motif scan and putative vicinal disulfide linkages. Alanine amino acid residues were substituted for amino acid residues to eradicate both known consensus sequences from the motif scan and alanine was substituted for various patterns of the cysteines (Figure 6).

3.3 General Overview of the Agonist-Dependent Internalization Assay

To determine what effect each of the different mutations has on the internalization elicited by the muscarinic receptor selective agonist carbachol,

agonist-dependent internalization assays were performed. Agonist activation of muscarinic receptors is known to cause a rapid redistribution (internalization) of receptors from the plasma membrane to an intracellular compartment (Koenig and Edwardson, 1997), and in a previous investigation, the agonist-dependent internalization of hM_1 receptors was determined to require a domain or domains in the third cytoplasmic loop (i3) of the receptor (Lameh *et al.*, 1992). This was consistent with the recently identified small domain (252-265) in the hM_1 receptor i3 loop that was shown to affect agonist-induced internalization of the receptor when deleted (unpublished observations). To completely characterize the role this small domain plays in the agonist-induced internalization of hM_1 receptors, CHO-K1 cells, which do not express muscarinic receptors, were transfected with various mutant pCD-hM_1 receptor constructs possessing different amino acid substitutions (see Figure 6). The transfected cells were allowed to produce and express receptor for 24 hours prior to treatment with carbachol (1 mM) for time periods up to 4 hours. The concentration of carbachol used in this investigation was obtained from previous *in vitro* studies (Lameh *et al.*, 1992; Lee *et al.*, 1998; Maeda *et al.*, 1990; Moro *et al.*, 1993; Tolbert and Lameh, 1996). Measurement of plasma membrane expressed receptors before and after carbachol treatment was accomplished using an intact whole cell binding assay with the membrane impermeable antagonist [^{3}H]NMS. Like carbachol, [^{3}H]NMS is selective for muscarinic receptors. Percent of maximal binding was calculated by dividing the fmol specific binding at a given time point with agonist stimulation by the fmol specific binding at the zero time point (no agonist). This internal standardization

allowed the hM_1/WT data previously obtained in the laboratory, using the same protocol as described in the "Research Methods" section, to be compared to current data.

3.4 General Overview of Saturation Radioligand Binding Assay

Saturation radioligand binding assays allow for the measurement of the maximal number of receptor binding sites (B_{max}), and the equilibrium dissociation constant (K_D) of a ligand. The B_{max} and K_D are valuable in receptor classification, and interpretations on possible changes in receptor structure that affect affinity. The emphasis of this investigation was to determine if the effects of various mutations in the i3 loop of hM_1 receptors on internalization were due to changes in affinity of the ligand for the receptor.

The affinity of a ligand for a receptor is defined by the interaction strength between the two and is quantified by the equilibrium dissociation constant. Distinctively, the K_D is the concentration of ligand that binds to 50% of the total receptor population. Correspondingly, the higher the K_D value, the lower the affinity of the ligand has for the receptor because it takes a higher concentration of ligand to bind 50% of the receptors. The maximal number of binding sites (B_{max}), is defined at theoretically infinite ligand concentrations and its accuracy is proportional to the maximal levels of radioligand that can be used in the experiment to determine receptor density (Kenakin, 2003). In this experiment, [^{3}H]NMS was used to define B_{max} of all the hM_1 receptor constructs characterized. [^{3}H]NMS is a high affinity radioligand for hM_1 ($pK_D = 9.57 \pm 0.07$).

Therefore, it was possible to obtain ~ 100% occupancy and B_{max} could be estimated from saturating radioligand binding assays using equation 5 (page 26).

3.5 General Overview of Phosphoinositide Hydrolysis Assay

hM_1 receptors transduce agonist binding by activating G proteins to regulate ion channel activity and/or generate second messengers by utilizing the phosphoinositide (PI) system. At basal state conditions, G proteins exist in cell membranes as heterotrimers composed of single α, β, and γ subunits, with GDP bound to the α subunit (Birnbaumer *et al.*, 1990; Siegal *et al.*, 1999). When an agonist binds the hM_1 receptor, the receptor physically associates with the α subunit. This interaction promotes the disassociation of GDP from the α subunit and GTP can bind in its place. The α subunit with GTP disassociates from the $\beta\gamma$ subunits, and regulates an effector protein, phospholipase C (PLC). Hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) by PLC yields two products: 1,2-diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP_3). Incubation of hM_1 transfected CHO-K1 cells with [^{3}H]inositol 24 hours before performing the assay allows incorporation of [^{3}H] into PIP_2. Therefore, to determine whether mutant hM_1 receptors could still elicit phosphatidylinositol metabolism when exposed to agonist, [^{3}H]IP_3, which does not recycle in the presence of 10 mM LiCl, was assayed and counted with a scintillation counter. Measurement of potency is defined using the EC_{50}, which is the molar concentration of an agonist required to produce 50% of the maximal response to the agonist.

3.6 Mutant Receptors hM_1/T-A and hM_1/P-A

To determine whether the effect on internalization, seen previously when the small domain (252-265) was deleted in the i3 loop of hM_1 receptors, was a consequence of disrupting either a putative SH3 binding domain or a proline dependent serine/threonine kinase site, mutations at critical positions for each consensus sequence were made. To disrupt the putative SH3 binding domain, an alanine was substituted at position 252 for proline (hM_1/P-A, see Figure 6) and alanine was substituted at position 254 for threonine (hM_1/T-A, see Figure 6) to disrupt the putative kinase site. Internalization, saturation binding and phosphoinositide hydrolysis assays were then performed.

3.6.1 Internalization of hM_1/T-A and hM_1/P-A Receptor Mutants

Figure 7 depicts the effects of carbachol (1 mM) treatment on the plasma membrane expression of hM_1/T-A and hM_1/P-A receptor mutants compared to wild-type hM_1 receptors. Approximately 40% of maximal specific [^{3}H]NMS binding was observed for hM_1/WT, T-A and P-A during the 4 hour carbachol treatment, suggesting that neither mutation had an effect on agonist-induced internalization (Table II). The percent internalization was very similar when comparing these mutant receptors to WT receptors (Table III). In fact, no significant differences ($F_{2,66} = 0.86$; $p > 0.05$) were observed at each of the time points considered (Table II, Figure 7) when using a two-way ANOVA with post hoc Bonferroni post-test with selective comparisons. Therefore, the effect on agonist-dependent internalization seen previously with the hM_1/8 aa del mutant

receptor, does not appear to be a result of disrupting either the putative SH3 domain or serine/threonine kinase site.

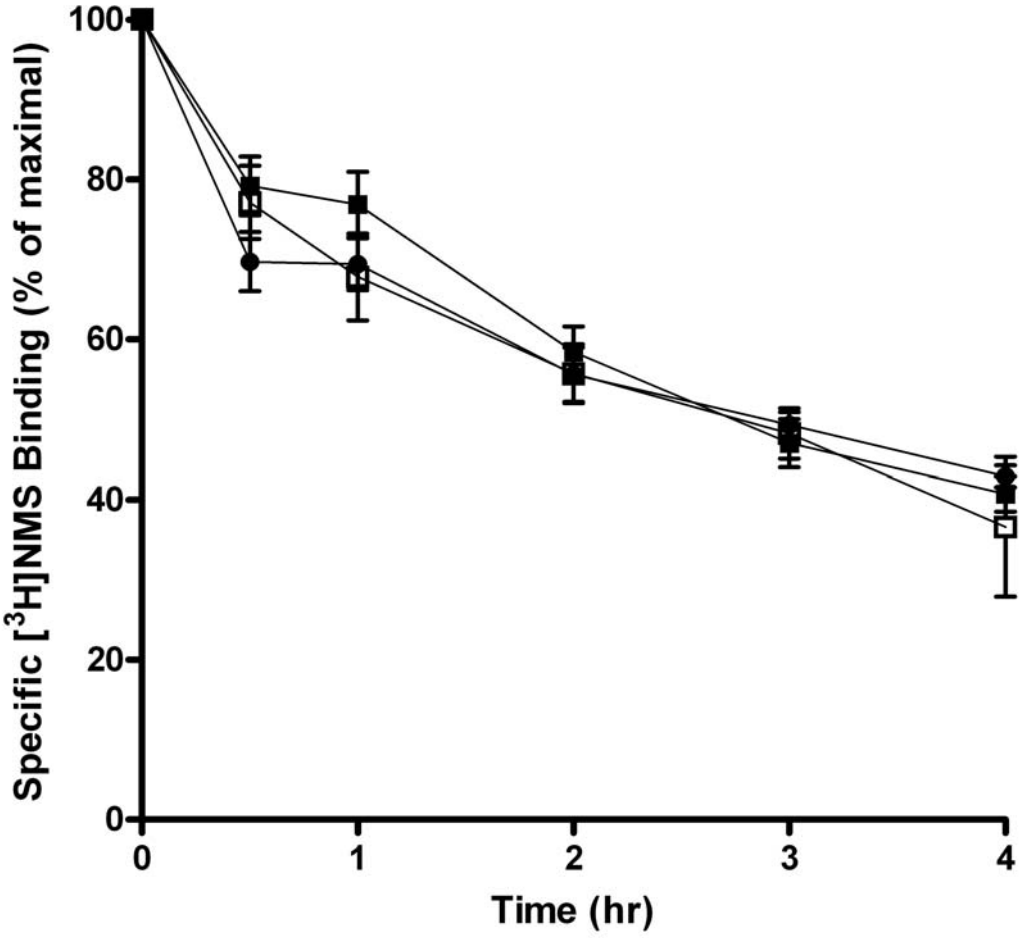

Figure 7. Carbachol-dependent internalization of hM_1/T-A and hM_1/P-A receptors. The extent of mutant hM_1/T-A and hM_1/P-A receptor internalization in response to the agonist carbachol (1 mM) showed no significant difference from the hM_1/WT receptor. CHO-K1 cells transiently expressing hM_1/T-A, or hM_1/P-A receptors were incubated with the agonist carbachol (1 mM) for up to 4 hr at 37°C in an atmosphere of 5% CO_2 before conducting intact whole cell [^{3}H]NMS binding assays (see "Research Methods"). The mutants hM_1/P-A (●), hM_1/T-A (■), and hM_1/WT (□) are shown. Each series of data points represents an experiment conducted in triplicate either 3 (hM_1/WT, hM_1/P-A), or 8 times (hM_1/T-A), ± S.E.M.

Table II. Summary of hM_1/WT, T-A and P-A Receptor [^{3}H]NMS Binding

Time of Carbachol (1mM) Treatment	Specific [^{3}H]NMS Binding (% of maximal) For Receptor Constructs		
	hM_1/WT[1]	hM_1/T-A[2]	hM_1/P-A[1]
0.5 hr	77.1 ± 4.6	79.2 ± 3.7	69.7 ± 3.7
1 hr	67.8 ± 5.4	76.9 ± 4.0	69.4 ± 3.2
2 hr	55.7 ± 3.7	58.4 ± 3.2	55.6 ± 3.4
3 hr	48.2 ± 3.1	47 ± 3.0	49.3 ± 1.6
4 hr	36.6 ± 8.7	40.7 ± 2.2	42.9 ± 1.4

hM_1/T-A and P-A receptor mutants internalize in a manner consistent with hM_1/WT receptors when incubated with carbachol for up to 4 hours. Data presented in table represents data points in Figure 7.

[1] Each time point data represents the mean ± S.E.M. of 3 experiments conducted in triplicate.

[2] Each time point data represents the mean ± S.E.M. of 8 experiments conducted in triplicate.

Table III. Percent Internalization of hM_1/WT, T-A, and P-A at Given Time Points

	% Internalization				
hM_1 Receptor Constructs	0.5 hr	1.0 hr	2.0 hr	3.0 hr	4.0 hr
T-A	20.8	23.1	41.6	53.0	59.3
P-A	30.3	30.6	44.4	50.7	57.1
WT	22.9	32.2	44.3	50.7	57.1

Data presented in Table III represents the percent internalization of hM_1 receptors based on the agonist-dependent internalization assays with [^{3}H]NMS.

3.6.2 hM_1/T-A and hM_1/P-A Dissociation Equilibrium Constant

To determine if the disruption of either the putative SH3 binding domain or the proline dependent serine/threonine kinase site affected binding of the ligand to the receptor, CHO-K1 cells were transiently transfected with hM_1/T-A and hM_1/P-A and then incubated with geometrically spaced (0.33 log unit) concentrations of [^{3}H]NMS as described in "Research Methods". Figure 8 depicts the nonlinear curve fitted plot of hM_1/T-A and P-A compared to hM_1/WT. The affinity or strength of interaction of the ligand to bind to the receptor was decreased significantly ($F_{2,8} = 17.84$, $p < 0.01$) between the hM_1/WT and hM_1/T-A and P-A. The pK_D for hM_1/WT was 9.57± 0.07 and the pK_D for hM_1/T-A and P-A was 9.22 ± 0.04 and 9.23 ± 0.04 respectively (Table IV). Even though the affinity of the ligand for the mutant receptors was lower, the ligand was not prevented from binding. This would suggest that the putative SH3 binding domain or proline dependent serine/threonine kinase site is not responsible for the decreased extent of agonist-dependent internalization observed for hM_1/8 aa del.

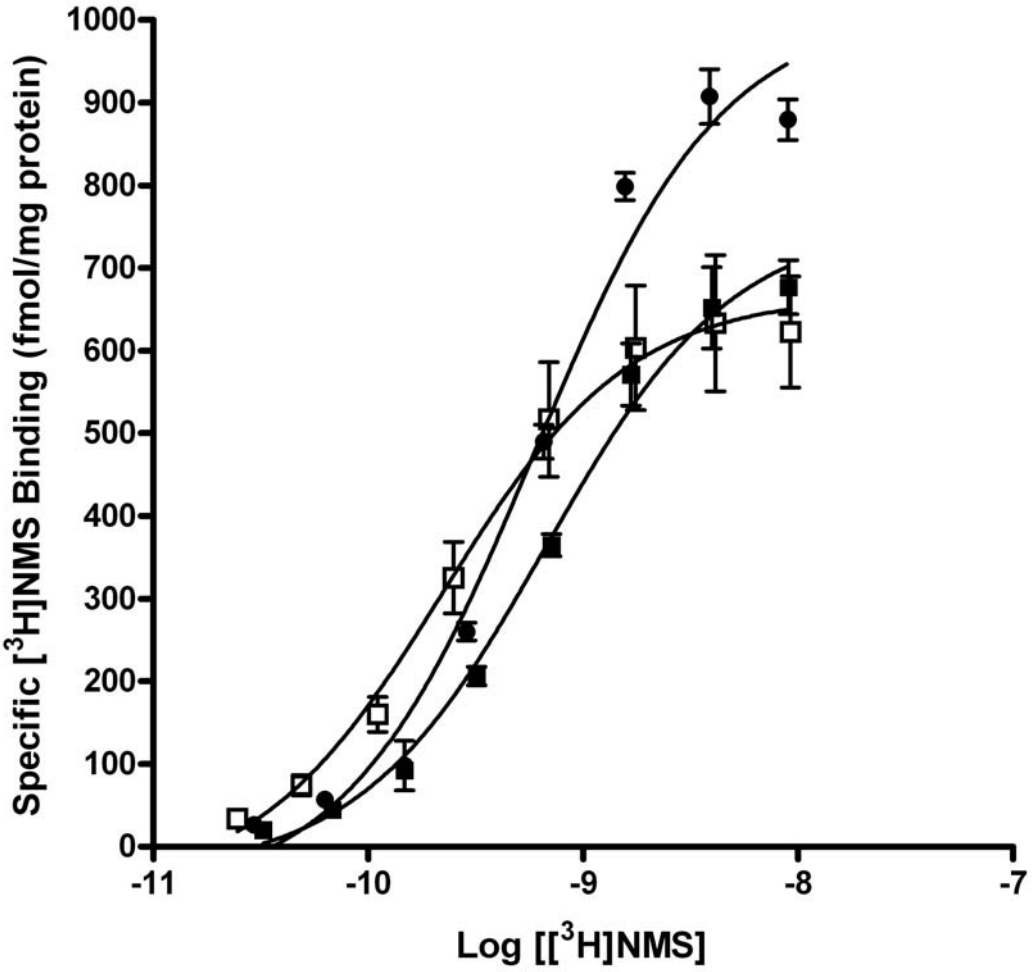

Figure 8. Specific [^{3}H]NMS binding comparison of hM_1/WT, hM_1/T-A, and hM_1/P-A receptors. CHO cells expressing either of the following hM_1 receptors: hM_1/ (□),hM_1/T-A (■), or hM_1/ P-A (●), were incubated with geometrically spaced (0.33 log unit) concentrations of [^{3}H]NMS as described in "Research Methods". Each data point represents the mean ± the S.E.M. of 3 experiments conducted in triplicate.

3.6.3 Maximal Number of Binding Sites (B_{max}) for hM_1/WT and hM_1/T-A and P-A Mutant Receptors

B_{max} describes the maximal number of binding sites available on the cell surface and can be defined when using saturating concentrations of the membrane impermeable [^{3}H]NMS. B_{max} indicates the amount of receptor density as molar concentration of receptors per milligram protein. The hM_1/T-A B_{max} values (685.80 ± 49.0) were similar to hM_1/WT (643.98 ± 69.9). There was a significant mutation dependent increase ($F_{2,8} = 7.687$, $p < 0.05$) in B_{max} between hM_1/P-A and hM_1/WT (Table IV).

Table IV. Mutant Receptors hM_1/T-A and P-A pK_D, B_{max} , and Hill Slope Data From Saturation Binding Assay[1]

hM_1 Receptor Constructs	pK_D	B_{max}	Hill Slope
WT (□)	9.57 ± 0.07	643.98 ± 69.9	1.48 ± 0.20
T-A (■)	9.28 ± 0.05 **	685.80 ± 49.0	1.47 ± 0.05
P-A (●)	9.23 ± 0.04 **	916.50 ± 33.5 *	1.77 ± 0.11

[1]Data presented in table represents data collected from saturation radioligand binding assays (Figure 8). The K_D (equilibrium dissociation constant), B_{max} (maximal number of binding sites available) and Hill slope was estimated using nonlinear regression as described previously by Bowen and Jerman, 1995. Significant difference is denoted (* = $p < 0.05$ and ** = $p < 0.01$) and was determined using an one-way ANOVA with post hoc Bonferroni's multiple comparison test.

3.6.4 Agonist Potency of hM_1/T-A and hM_1/P-A Mutant Receptors

To determine if hM_1/T-A and P-A receptors could still elicit phosphatidylinositol metabolism when exposed to agonist, CHO-K1 cells were transiently transfected with mutant receptor and phosphoinositide hydrolysis (PI) assays were performed as described in “Research Methods”.

The concentration of the agonist carbachol that produced 50% of the maximal response for mutant hM_1/T-A (pEC_{50} = 5.57 ± 0.05) and hM_1/P-A (pEC_{50} = 5.46 ± 0.03) was similar to hM_1/WT (pEC_{50} = 5.34 ± 0.10). The mutations did not affect the potency of phosphoinositide hydrolysis elicited to carbachol (Figure 9), but there was a 1.6-fold increase in maximal response for both hM_1/T-A and P-A compared to hM_1/WT.

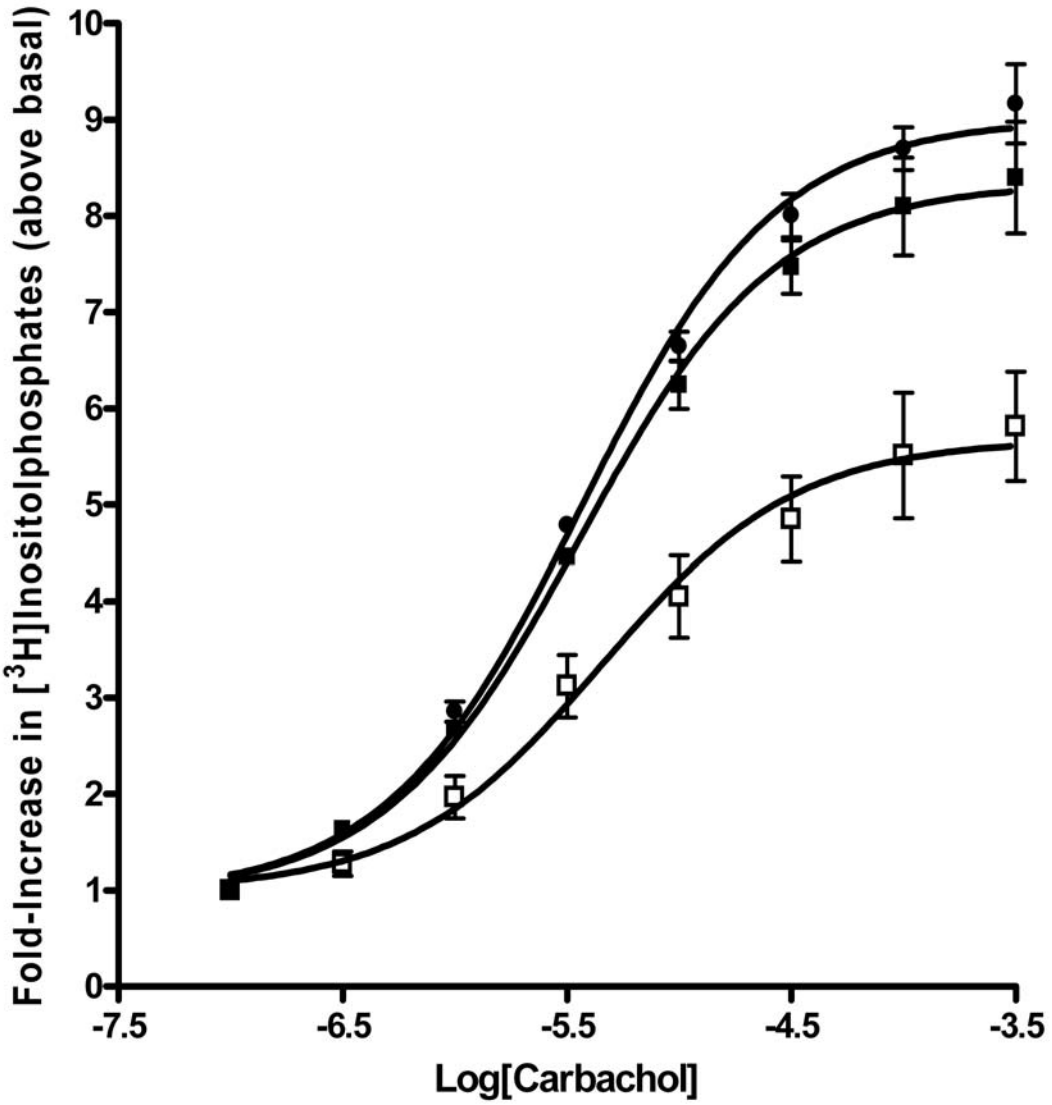

Figure 9. Comparison of the phosphoinositide response of hM_1/WT, hM_1/T-A and hM_1/P-A receptors. hM_1/T-A (■) and hM_1/P-A (●) mutations did not affect the potency of PI hydrolysis elicited to the agonist carbachol. CHO-K1 cells transiently expressing hM_1/T-A, or hM_1/P-A receptors were incubated with [^{3}H]*myo*-inositol (0.2 μM) 24 hours before performing phosphoinositide hydrolysis assays (see "Research Methods"). Each data point represents an experiment conducted in triplicate either 3 or 4 times ± the S.E.M.

3.7 Characterization of Mutant Receptors hM_1/2C-A, hM_1/2CC, hM_1/4CC and hM_1/CC-AA

To determine whether the effect on internalization, seen previously when the small domain (252-265) was deleted in the i3 loop of hM_1 receptors, was due to vicinal disulfide bonds or other effects of cysteine residues, mutations substituting alanine for various cysteines were made. Vicinal disulfide bonds may form between adjacent cysteine residues, so mutations changing the cysteines 259 and 260 (hM_1/CC-AA), 261 and 262 (hM_1/2CC), and 259, 260, 261, and 262 (hM_1/4CC), and 262 (hM_1/2C-A) to alanine(s) were made (Figure 6).

3.7.1 Internalization of Cysteine to Alanine Receptor Mutants

Figure 10 summarizes the effects of carbachol (1 mM) on the plasma membrane expression of hM_1/CC-AA, hM_1/2CC, hM_1/4CC, hM_1/2C-A receptor mutants compared to hM_1/WT. In general, the cysteine to alanine mutations had a significant ($F_{4,96}$ = 99.04, $p < 0.0001$) affect on the agonist-dependent internalization of mutant receptor when compared to WT.

Multiple comparisons between mutated receptors were performed using Bonferroni's multiple comparison tests. Among the cysteine to alanine mutations tested the least responsive to carbachol-induced internalization were the hM_1/4CC and hM_1/2CC receptors. These receptors are very similar to each other. The 2CC and 4CC receptors are both mutated at amino acids 262 and 263 (Figure 6). Compared to the hM_1/WT receptor starting at time point 1 hour thru the 4 hour time point there was significant difference ($p < 0.001$) from both

the hM_1/4CC and hM_1/2CC receptors in the amount of specific [^{3}H]NMS binding (Table V).

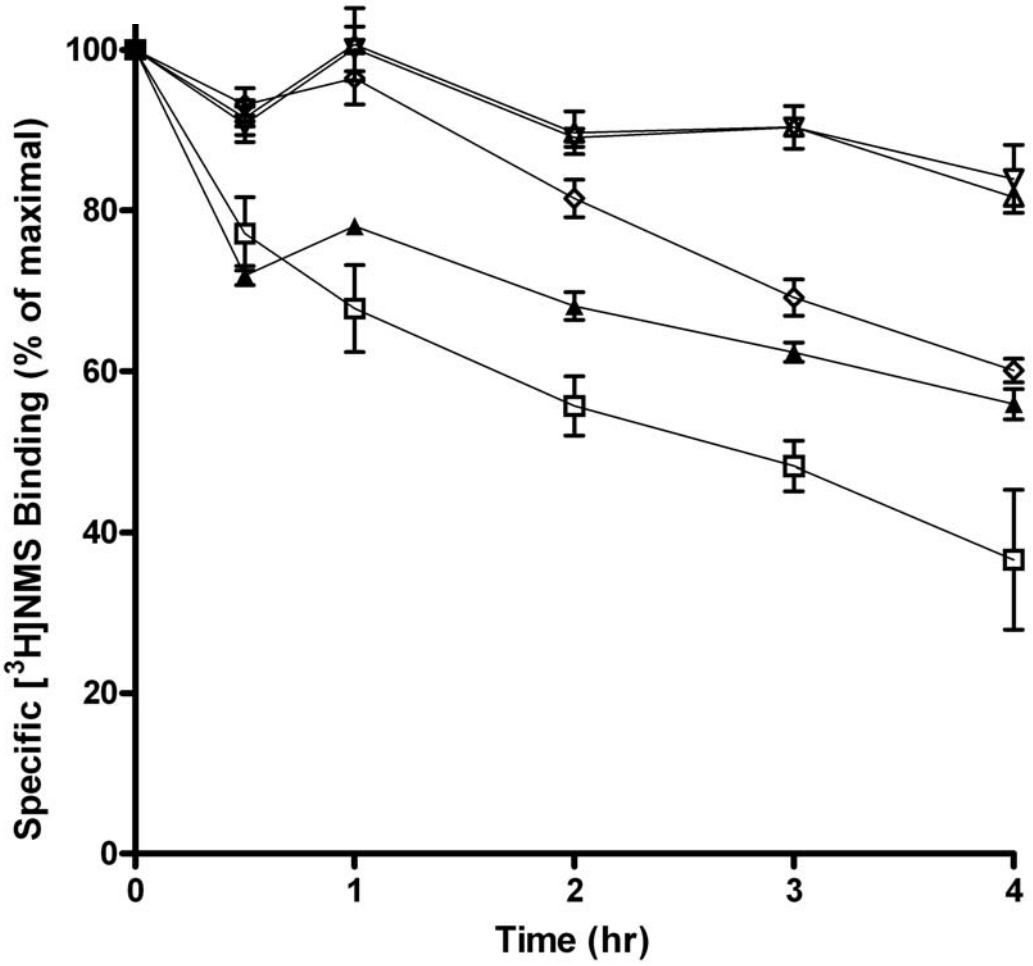

Figure 10. Carbachol-dependent internalization of cysteine to alanine mutant receptors. The extent of mutant hM_1 receptor internalization varies depending upon the position of the mutation within the i3 loop domain 252-265 (Figure 6). CHO-K1 cells transiently expressing various cysteine to alanine mutant hM_1 receptors were incubated with the agonist carbachol (1 mM) for up to 4 hours at 37°C in an atmosphere of 5% CO_2 before conducting whole cell [^{3}H]NMS binding assays (see "Research Methods"). The hM_1/WT (□), and mutants hM_1/2C to A (▲), hM_1/CC-AA (◇), hM_1/2CC (△), hM_1/4CC (▽), are shown. Each data series represents an experiment conducted in triplicate either 8 (hM_1/CC-AA), 4 (hM_1/4CC), or 3 times (hM_1/WT, hM_1/2C-A, and hM_1/2CC,), ± the S.E.M.

hM_1/2C-A internalization in response to the agonist carbachol showed no significant difference from the hM_1/WT receptor for the time points 0.5 hour, 1 hour, 2 hour, however the 3 hour and 4 hour time point did show significant decrease ($p < 0.05$ and $p < 0.001$ respectively) in extent of internalization from hM_1/WT (Table V). hM_1/CC-AA showed significant decrease ($p < 0.001$) in extent of internalization from hM_1/WT for all time points (Table V). Both hM_1/2C-A and hM_1/CC-AA displayed partially deficient internalization when compared to hM_1/4CC and 2CC (Figure 10). The hM_1/CC-AA mutant receptor is very similar in percent internalization to the mutants hM_1/4CC and 2CC at the 0.5 hour, 1 hour, and 2 hour time point. However, there was a 3.2-fold increase in internalization of the hM_1/CC-AA receptor at the 3 hour time point and ~2.3-fold increase at the 4 hour time point when compared to hM_1/4CC and 2CC. It is interesting to point out that the mutant receptor hM_1/CC-AA is similar in sequence to the hM1/4CC because they are both mutated at amino acids 259 and 260 (Figure 6). The decrease in [^{3}H]NMS binding is not as extensive as the hM_1/WT receptor at the 3 hour time point where internalization of hM_1/CC-AA is 1.6-fold less than WT, and 1.4-fold less at the 4 hour time point (Table VI). Similarly, the hM_1/2C-A receptor does not internalize to the extent of the hM_1/WT at time point 3 hour (1.3-fold less) and 4 hour (1.3-fold less). It is interesting to note the 3.8-fold greater internalization of hM_1/2C-A at the 0.5 hour time point when compared to hM_1/CC-AA but at the 4 hour time point there is only 1.1-fold difference in internalization (Table VI) .

Table V. Summary of hM_1/WT, 4CC-AA, 2CC-AA, 2C-A, and CC-AA Receptor [^{3}H]NMS Binding

	Specific [^{3}H]NMS Binding (% of maximal) For Receptor Constructs[1]				
Time of Carbachol (1mM) Treatment	hM_1/WT[2]	hM_1/4CC[2]	hM_1/2CC[2]	hM_1/2C-A[2]	hM_1/CC-AA[3]
0.5 hr	77.1 ± 4.6	90.7 ± 2.2*	91.6 ± 2.2*	71.9 ± 1.2	93.2 ± 2.1**
1 hr	67.8 ± 5.4	100 ± 2.8**	100 ± 4.5**	78 ± 1.0	96.5 ± 3.3**
2 hr	55.7 ± 3.7	89.1 ± 1.1**	89.7 ± 2.7**	68.1 ± 1.7	81.5 ± 2.3**
3 hr	48.2± 3.1	90.4 ± 2.7**	90.4 ± 1.0**	62.4 ± 1.2*	69.2 ± 2.3**
4 hr	36.6 ± 8.7	83.9 ± 4.2**	81.7 ± 0.9**	55.9 ± 1.9**	60.1 ± 1.5**

[1]Significant differences were determined by using a two way ANOVA with post hoc Bonferroni post-test selective comparisons between hM_1 mutants and hM_1/WT for each time point. * denotes significant difference ($p<0.05$), and ** denotes significant difference ($p < 0.001$) from hM_1/WT. Data presented in table represents data points from Figure 10.

[2] Each data time point represents the mean ± S.E.M. of 3 experiments conducted in triplicate.

[3] Each data time point represents the mean ± S.E.M. of 8 experiments conducted in triplicate.

Table VI. Percent Internalization of hM_1 Cysteine to Alanine Mutant Receptors at Given Time Points[1]

	% Internalization at the Specified Incubation Time				
hM_1 Receptor Constructs	0.5 hr	1.0 hr	2.0 hr	3.0 hr	4.0 hr
4CC	9.3	0.0	10.9	9.6	16.1
2CC	8.4	0.0	10.3	9.6	18.3
2C-A	25.5	22.5	32.9	38.2	45.0
CC-AA	6.7	3.5	18.5	30.8	39.9
WT	22.9	32.2	44.3	50.7	57.1

[1]Data presented in table represents the percent internalization of hM_1 receptors based on the agonist-dependent internalization assay with [^{3}H]NMS.

3.7.2 hM_1/2C-A, hM_1/2CC, hM_1/4CC, hM_1/CC-AA and hM_1/WT Dissociation Equilibrium Constant

To measure the equilibrium dissociation constant (K_D) to determine if changing the cysteine residues to alanines effected the binding of the ligand to the receptor compared to hM_1/WT, CHO-K1 cells were transiently transfected with hM_1/2C-A, hM_1/2CC, hM_1/4CC, and hM_1/CC-AA. Transfected cells were then incubated with geometrically spaced (0.33 log unit) concentrations of [^{3}H]NMS as described in "Research Methods".

In general, the cysteine to alanine mutant receptors pK_D values were significantly ($F_{5,19} = 12.33$, $p < 0.0001$) decreased compared to hM_1/WT using an one way ANOVA. Figure 11 depicts the nonlinear curve fitted plot of the mutant hM_1 receptors compared to hM_1/WT. The pK_D values for hM_1/2CC, 4CC, and CC-AA, were decreased (hM_1/2CC and 4CC $p < 0.001$, hM_1/CC-AA $p < 0.01$) compared to hM_1/WT, and mutant receptors had a lower affinity (4.2-fold, 4.5-fold, 3.4-fold, respectively) of the ligand for the receptor (Table VII). hM_1/2C-A pK_D value was not significantly different than hM_1/WT, but did have a 1.9-fold lower affinity of the ligand for the receptor (Table VII).

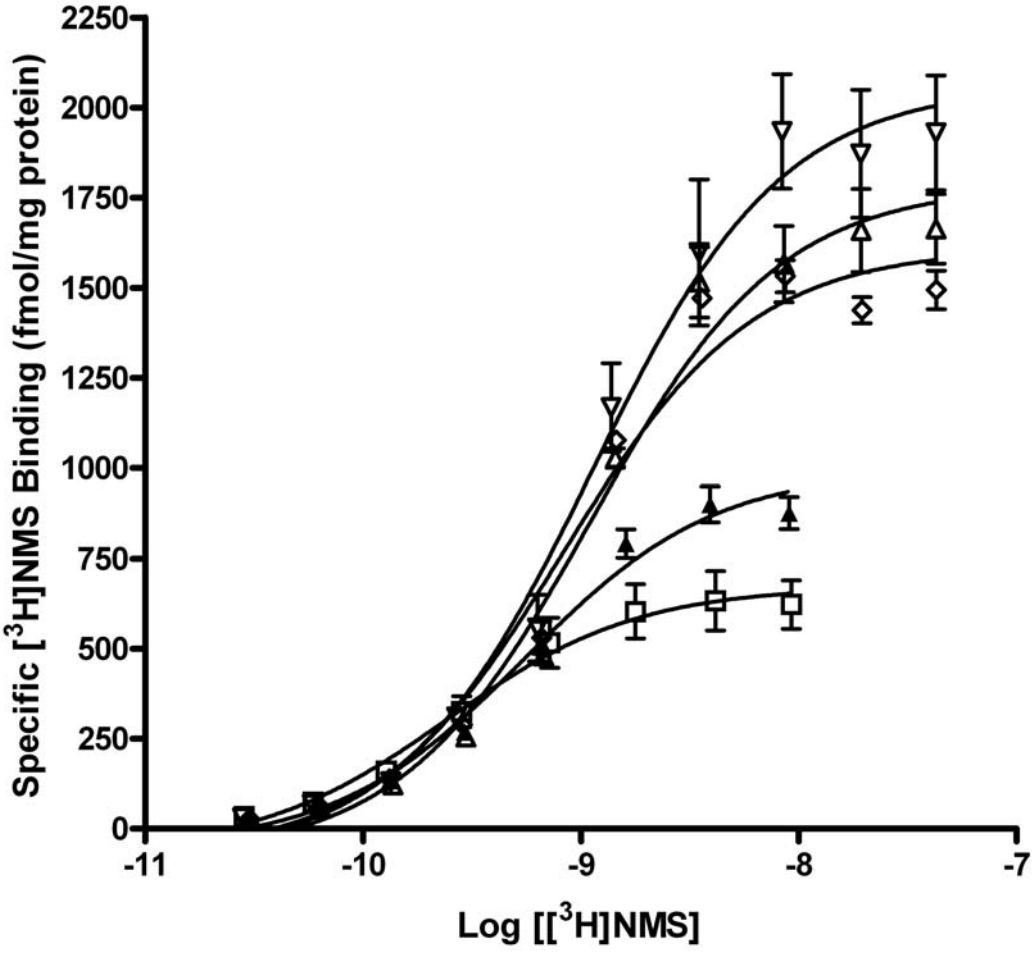

Figure 11. Cysteine to alanine mutant receptor comparison of specific [^{3}H]NMS binding to hM_1/WT receptor. CHO-K1 cells transiently transfected with one of the following, hM_1/2C-A (▲), hM_1/2CC(△), hM_1/4CC(▽), hM_1/CC-AA(◇), hM_1/WT(□), were incubated with geometrically spaced (0.33 log units) concentrations of [^{3}H]NMS as described in "Research Methods". Each data point represents the mean ± the S.E.M. of 3 experiments conducted in triplicate.

Table VII. pK_D, B_{max} , and Hill Slope Data From Saturation Binding Assay of hM_1 Cysteine to Alanine Mutant Receptors[1]

hM_1 Receptor Constructs	pK_D	B_{max}	Hill Slope
WT (□)	9.57 ± 0.07	643.98 ± 69.9	1.48 ± 0.20
2C-A (▲)	9.28 ± 0.05	885.06 ± 75.3	1.73 ± 0.07
2CC (△)	8.95 ± 0.02*	1664.26 ± 102.3 *	1.82 ± 0.26
4CC (▽)	8.92 ± 0.10*	1983.78 ± 162.1 *	1.57 ± 0.15
CC-AA (◇)	9.04 ± 0.04**	1511.87 ± 17.1 **	1.96 ± 0.26

[1]Data presented in table represents data collected from saturation radioligand binding assay (Figure 11). The K_D (equilibrium dissociation constant), B_{max} (maximal number of binding sites available) and Hill slope was estimated using nonlinear regression as described previously by Bowen and Jerman, 1995. Significantly different from WT (* denotes $p < 0.001$)(** denotes $p < 0.01$) was determined using a one way ANOVA and post hoc Bonferroni's multiple selective comparison test.

3.7.3 Maximal Number of Binding Sites (B_{max}) for Cysteine to Alanine Mutant Receptors

In general, cysteine to alanine mutant receptors B_{max} values were significantly ($F_{5,19}$ = 18.45, $p < 0.0001$) increased compared to hM_1/WT using a one way ANOVA. Mutant receptors, hM_1/2CC, 4CC, and CC-AA, exhibited greater receptor expression on the cell surface compared to hM_1/WT (Table VII). In this experiment this increase in expression on the cell surface is consistent with the receptor internalization data because the mutant receptor extent of internalization was lower leaving more receptors on the cell surface. For example, with hM_1/2CC and 4CC the maximum receptor percent internalization was 18.3% (Table VI) leaving the majority of the receptors on the cell surface for the ligand to bind, correlating to the increase in maximal number of binding sites available. hM_1/CC-AA (B_{max} = 1511.87 ± 17.1) had a deficient internalization response therefore the increase in maximal number of binding sites compared to hM_1/WT (B_{max} = 643.98 ± 69.9) would be expected as well as the maximal number of binding sites available be less than hM_1/2CC, and 4CC.

Mutant receptor hM_1/2C-A (B_{max} 885.06 ± 75.3) has a maximal number of binding sites compared to hM_1/WT (B_{max} 643.98 ± 69.9). Comparing the four mutant receptors made by changing cysteine(s) to alanine(s), hM_1/2C-A internalized most similar to hM_1/WT, therefore expected maximal binding sites available would be similar.

3.7.4 Agonist Potency of Cysteine to Alanine Mutant Receptors

To ascertain if the reduced agonist-dependent internalization of the cysteine to alanine receptor mutants was a consequence of loss of function of the receptor, CHO-K1 cells were transiently transfected with hM_1/2CC, 4CC, CC-AA, or 2C-A and phosphoinositide hydrolysis (PI) assay was performed as described in “Research Methods”.

In general, all cysteine to alanine mutant receptors were similar to hM_1/WT in potency with no significant difference as determined using an one-way ANOVA. The cysteine to alanine mutant receptor that had the most similar response to hM_1/WT was hM_1/2C-A with pEC_{50} of 5.34 ± 0.10 and 5.47 ± 0.05, respectively. All cysteine to alanine mutant receptors caused an increase in the maximal response of the receptor induced by the agonist (Figure 12).

Table VIII. Potency of Cysteine to Alanine Mutant Receptors

hM_1 Receptor Constructs	pEC_{50}
WT	5.34 ± 0.10
4CC	5.67 ± 0.06
2CC	5.60 ± 0.06
2C-A	5.47 ± 0.05
CC-AA	5.62 ± 0.03

Data presented in table represents data collected from phosphoinositide hydrolysis assays (Figure 12). The pEC_{50} was estimated using nonlinear regression as described previously by Bowen and Jerman, 1995.

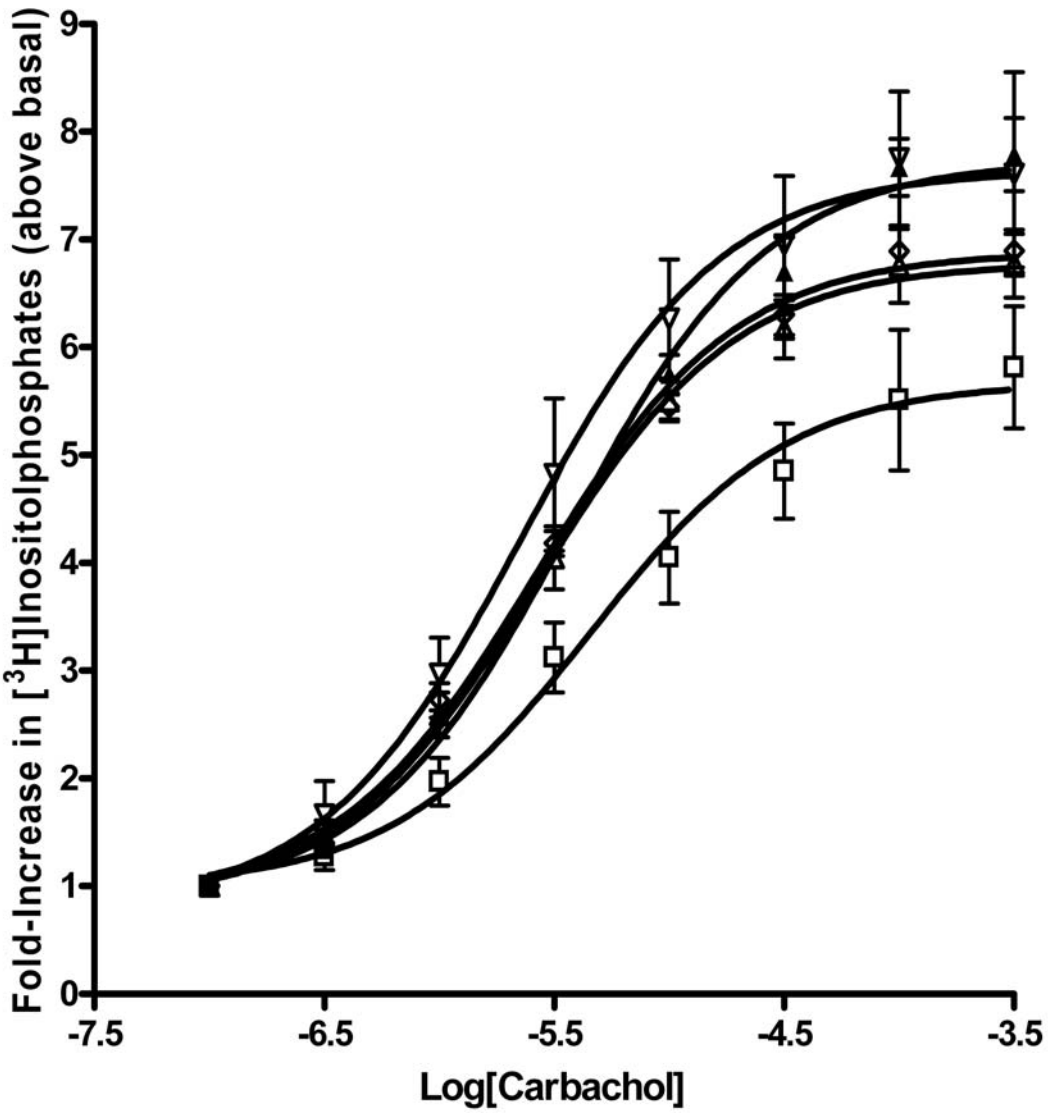

Figure 12. Cysteine to alanine mutant receptor comparison of phosphoinositide response to hM_1/WT receptor. hM_1/2C-A (▲), hM_1/2CC (△), hM_1/4CC (▽), and hM_1/CC-AA(◇) mutant receptors are shown in comparison to hM_1/WT (□). CHO-K1 cells transiently expressing hM_1 mutant receptors were incubated with [^{3}H]*myo*-inositol (0.2 μM) 24 hours before performing phosphoinositide hydrolysis assay (see "Research Methods"). Each data point represents an experiment conducted in triplicate either 3 or 4 times, ± the S.E.M

Chapter IV

Discussion

In a previous study, deletion of the 8 amino acid domain 254-TPPGRCCR-262 from hM_1 receptors was determined to decrease the extent of agonist-dependent hM_1 receptor internalization. This decrease in internalization could not be attributed to a lack of ligand binding or loss of receptor signaling as determined in intact cell radioligand binding and phosphoinositide hydrolysis assays, respectively (unpublished results). The primary objective of this study was to further these previous observations by trying to identify specific amino acid residues within or adjacent to this 8 amino acid domain that play a role in agonist-dependent hM_1 receptor internalization. To accomplish this objective, various amino acid residues were mutagenized within the domain 252-PETPPGRCCRCC-263 of the hM_1 receptor. Each of the resulting hM_1 receptor mutants was then characterized to determine the affect of the mutation on agonist-dependent internalization, ligand binding, and receptor signaling.

4.1 Dimerization

The previous study, which identified the 8 amino acid domain in the i3 loop of hM_1 receptors, focused on trying to determine whether or not domains of the

hM_1 receptors were capable of interacting with one another to possibility mediate hM_1 receptor dimerization. Growing evidence supporting the postulate that muscarinic receptors dimerize under certain conditions was the incentive for the previous investigation. To date, evidence for muscarinic receptor dimerization has been obtained in investigations using M_2 and M_3 receptor subtypes (Maggio et al., 1996; Park et al., 2001; Zeng and Wess, 2000; Zeng and Wess, 1999).

Using receptor chimeras composed of various segments of α_2-adrenergic and M_3-muscarinic receptors, it was established that GPCRs can interact with one another to form dimers (Maggio et al., 1996). When a chimeric receptor (α_2/M_3) composed of transmembrane domains one through five of α_2-adrenergic receptors fused with transmembrane domains six and seven of M_3-muscarinic receptors was expressed in CHO cells, no [^{3}H]NMS (muscarinic receptor-selective radioligand) or [^{3}H]rauwolscine (adrenergic receptor-selective radioligand) binding was observed (Maggio et al., 1996). A similar result was obtained for a receptor chimera composed of transmembrane domains one through five of M_3-muscarinic receptors fused with transmembrane domains six and seven α_2-adrenergic receptors (M_3/α_2). Interestingly, when these receptors were coexpressed (i.e., α_2/M_3 and M_3/α_2) in CHO cells, both [^{3}H]NMS and [^{3}H]rauwolscine binding was observed (Maggio et al., 1996). These observations suggested that the α_2/M_3 and M_3/α_2 receptor chimeras were dimerizing and the i3 loop of the M_3 receptor was determined to mediate the interaction between the receptors (Maggio et al., 1996). This also suggested that the i3 loop of M_3 receptors could mediate dimerization of M_3 receptors alone, a postulate

investigated by Zeng and Wess (1999). They determined that the i3 loop of M_3 receptors did not mediate M_3 receptor dimerization, but cysteine residues in the extracellular loops of the M_3 receptor did (Zeng and Wess, 1999). One possible explanation for the contrast in results could be that lack of functional complementation seen by Maggio (1996) was due to steric hindrance, preventing exchange of the domains between the two chimeric receptors (Zeng and Wess, 2000). More recently, M_2 receptors were found to dimerize when expressed in *Sf*9 cells, although the domain mediating the interaction has not been described to date (Park et al., 2001).

Along with the evidence for muscarinic receptor dimerization, other GPCRs have been observed to form dimers and dimerization of these receptors has been demonstrated to affect internalization. Specifically, heterodimerization between opioid-δ and -κ receptors caused an increase in the rate of internalization when exposed to agonist (Jordan and Devi, 1999). β_2-adrengeric and opioid-receptor heterodimers have been observed to form and the consequence of this dimerization was to affect trafficking of both receptors. Therefore, the possibility that deleting 254-TPPGRCCR-262 from the i3 loop of hM_1 receptors prevents hM_1 receptor dimerization, resulting in decreased hM_1 receptor internalization, exists. In future investigations, this possibility would be addressed by conducting co-immunoprecipitation studies using differentially tagged wild-type and mutant hM_1 receptors to verify that wild type receptors dimerize and whether or not deletion of 254-TPPGRCCR-262 prevents dimerization.

4.2 Phosphorylation of hM_1 Receptors

A protein sequence scan performed on the domain 254-TPPGRCCR-262 and surrounding amino acid residues showed that a putative proline-dependent serine/threonine kinase phosphorylation site overlapped with the domain. Phosphorylation of GPCRs often leads to internalization. Specifically, M_1 receptors have been shown to be phosphorylated at a specific serine- and threonine-rich site in the i3 loop by protein kinases in an agonist-dependent manner that is temporally consistent with removal of the receptor from the plasma membrane (Haga et al., 1996). Once phosphorylated, arrestin may bind the phosphorylated domain, promoting the internalization of the receptor in clathrin-coated pits (Wu et al., 1997; Lefkowitz and Shenoy, 2005). To date, the interaction between muscarinic receptors and arrestins have not been completely described and more research needs to be done to understand the mechanism by which arrestins mediate muscarinic receptor internalization.

To determine whether the putative proline-dependent serine/threonine kinase phosphorylation site identified plays a role in agonist-dependent hM_1 receptor internalization, a mutant hM_1 receptor was made lacking the consensus sequence (i.e., hM_1/T-A, see Figure 6) and then characterized. As seen in Figure 7 and Tables II and III, there was no difference in the carbachol-induced internalization of the hM_1/T-A receptor mutant when compared to hM_1-WT. This observation suggests that the disruption of this putative phosphorylation site caused by deleting the domain 254-TPPGRCCR-262 from the i3 loop of hM_1

receptors could not account for the decreased extent of carbachol-induced internalization of hM_1/8aa del (see Figure 4).

4.3 SH3 Binding Domain

Some GPCRs are known to contain SH3 domains, also known as SH3 ligands, and can interact with proteins that possess SH3 binding domains which recognize the SH3 domain consensus sequence PXXP (Ren *et al*., 1993). The protein motif scan conducted at the beginning of this investigation identified a putative SH3 domain overlapping with the domain 254-TPPGRCCR-262 (see Figure 6). To determine whether the deletion of 254-TPPGRCCR-262 from hM_1/8aa del disrupted this putative SH3 domain, resulting in the decreased extent of carbachol-induced hM_1 receptor internalization, a proline to alanine point mutation was made to disrupt the putative site (Figure 6) and the resulting mutant (hM_1/P-A) was characterized. As seen in Figure 7 and Tables II and III, the hM_1/P-A receptor mutant internalized in a manner similar to that observed for hM_1-WT. This observation suggests that the putative SH3 domain in the i3 loop of hM_1 receptors does not play a role in carbachol-induced internalization.

4.4 Cysteine to Alanine hM_1 Receptor Mutants

Muscarinic receptor expression at the plasma membrane is based on an equilibrium that is established between pathways that bring the receptor to the cell surface and back into the intracellular realm. Endocytosis refers to a diverse set of mechanisms that mediate receptor internalization, which often follows

receptor desensitization and can lead to receptor down-regulation. Removal of the putative phosphorylation site and the SH3 binding domain did not affect hM_1 receptor internalization (see Figure 7). This led us to speculate that the deletion of cysteine residues 259 and 260 in hM_1/8aa del resulted in the decreased hM_1 receptor internalization seen previously (see Figures 4 and 6). Therefore, the focus of this study moved to mutagenizing the cysteine residues within and surrounding the domain 254-TPPGRCCR-262 of hM_1 receptors.

4.4.1 Internalization of Cysteine to Alanine hM_1 Receptor Mutants

Two mutant hM_1 receptors were made possessing cysteines 262 and 263 mutated to alanines (i.e., hM_1/2CC and hM_1/4CC; see Figure 6) and then characterized. Both hM_1/2CC and hM_1/4CC receptor mutants were observed to have significantly different carbachol-induced internalization profiles when compared to hM_1-WT (see Figure 10). The hM_1/4CC receptor mutant also had cysteine residues 259 and 260 mutagenized to alanines. One other mutant, hM_1/CC-AA (see Figure 6), had this pair of cysteines mutagenized. The hM_1/CC-AA receptor mutant was also observed to have a significantly different carbachol-induced internalization profile when compared to hM_1-WT (see Figure 10). Overall, the data suggests that cysteine pairs 259-260 and 262-263 play important, but distinct roles in carbachol-induced hM_1 receptor internalization. This postulate is supported by the experiments performed on all of the cysteine to alanine receptor mutants made and is further described below.

The amount of receptor that internalized at the 0.5 hour time point was found to vary considerably between hM_1-WT and the cysteine to alanine receptor mutants (see Figure 10 and Tables V and VI). In general, the mutant receptors can be divided into two categories depending upon the extent of receptor internalization at the 0.5 hour time point. The hM_1/2C-A receptor mutant was observed to internalize in a manner consistent with hM_1-WT receptors. As seen in Figure 10, approximately 25% of total plasma membrane expressed hM_1/2C-A receptor internalized after 0.5 hour incubation with carbachol (1 mM); consistent with approximately 22% of total plasma membrane expressed hM_1-WT receptor. In contrast, very few hM_1/CC-AA, hM_1/2CC, and hM_1/4CC receptors internalized after 0.5 hour incubation with carbachol (see Figure 10 and Tables V and VI). Approximately 3% of total hM_1/CC-AA, hM_1/2CC, and hM_1/4CC mutant receptor expressed at the plasma membrane internalized at the 0.5-hour time point. Collectively, this data suggest that cysteine pair 259-CC-260 plays a role in this initial phase (i.e., 0 - 0.5 hour) of carbachol-induced hM_1 receptor internalization. The exact role that cysteine pair 259-CC-260 plays in internalization is not known at this time, but we suspect that it may be forming disulfide linkages with the adjacent cysteine pair 262-CC-263 to stabilize an important 2^o structure of the i3 loop that is necessary for this initial phase of internalization to occur.

This postulate is supported by the internalization of hM_1/CC-AA, which lacks cysteine pair 259-CC-260 but has cysteine pair 262-CC-263 (see Figure 6). As seen in Figure 10, the hM_1/CC-AA receptor mutant does not have an initial phase of internalization, which is similar to the hM_1/2CC receptor mutant.

hM_1/2CC has cysteine pair 259-CC-260, but does not have cysteine pair 262-CC-263 (see Figure 6). This suggests that the cysteine pairs are interacting with one another since the loss of either one result in the loss of the initial phase of carbachol-induced internalization. The rescue of the initial phase of hM_1 receptor internalization by the presence of cysteine 263 (i.e., hM_1/2C-A receptor mutant; see Figure 6) further strengthens this claim. As seen in Figure 10, the initial phase of carbachol-induced internalization is present for hM_1/2C-A and is similar to that observed for hM_1-WT. So it would seem that the presence of a single cysteine in the pair 262-CC-263 is adequate for cysteine pair 259-CC-260 to form a bond. This could be tested in a future study by creating an hM_1 receptor mutant that has only cysteine 262.

The extent of internalization of the hM_1/2CC and hM_1/4CC receptor mutants was also significantly different from the hM_1-WT receptor during the later phase (i.e., 0.5 hour – 4 hour) of carbachol-induced internalization (see Figure 10 and Tables V and VI). These observations suggest that cysteine pair 262-CC-263 is necessary for this phase of hM_1 internalization. Perhaps this pair of cysteine residues not only stabilizes a kink in the i3 loop of hM_1 receptors by bonding cysteine pair 259-CC-260, but also is recognized by the internalization machinery of CHO cells. Consequently, if the internalization machinery is prevented from binding the i3 loop of hM_1 receptors by deleting cysteine pair 262-CC-263, the receptor would not internalize when exposed to carbachol. The internalization of the hM_1/CC-AA receptor mutant supports this postulate (see Figure 10). hM_1/CC-AA lacks the cysteine pair 259-CC-260, but has cysteine

pair 262-CC-263 (see Figure 6). As seen in Figure 10, it lacks the initial phase of internalization as described above, but has a later phase of internalization like hM_1-WT.

In further support of our observations, a disulfide bond between cysteine residues 140 and 220, connecting the first and second extracellular loops of adjacent M_3 receptors has been described (Zeng and Wess, 2000). This demonstrates that even nonadjacent cysteine residues can bond with one another. Disulfide bonds between adjacent cysteine residues have also been described in a variety of proteins. This type of bonding has been shown to cause a tight turn, or kink, in a polypeptide and the conformation of the kink has been shown to change depending upon the cellular environment (Carugo *et. al*., 2003). Perhaps this is the mechanism by which cysteine pairs 259-CC-260 and 262-CC-263 play a role in carbachol-induced hM_1 receptor internalization. This possibility needs to be addressed in future studies.

4.4.2 Affinity and Potency of Cysteine to Alanine hM_1 Receptor Mutants

The binding of each mutant hM_1 receptor was characterized and compared to that of hM_1/WT receptor. This was done to ensure that the affect of the various mutations on carbachol-induced internalization was not a consequence of the ligand being prevented from binding to the receptor. As seen in Figure 11 and Table VII, all cysteine to alanine receptor mutants bound the muscarinic receptor-selective radioligand [^{3}H]NMS. This suggests that none of the mutations prevented ligand binding, thus resulting in altered receptor

internalization. However, almost all of the cysteine to alanine point mutations made, caused a significant increase in the pK_D values obtained from saturation binding assays when compared to hM_1-WT (see Table VII). While this increase was significant, it only amounted to an approximate 3-fold increase in pK_D value. The pK_D determined for carbachol binding hM_1-WT receptors is approximately 6 when the assay is conducted in the presence of guanine nucleotide (e.g., a condition similar to the carbachol-induced internalization assay performed on intact cells; unpublished observation). Assuming that the affect of each cysteine to alanine mutation on [^{3}H]NMS binding is similar to that for carbachol binding, near maximal receptor occupancy would be obtained for each receptor mutant, and would be comparable to that for hM_1-WT receptors, at the concentration of carbachol used (1 mM).

In support of the above statement, the pEC_{50} values obtained for phosphoinositide hydrolysis elicited to carbachol for all cysteine to alanine hM_1 receptors (pEC_{50} = ~5.5) was comparable to that obtained for hM_1-WT receptors pEC_{50} = 5.34 $\pm$ 0.1; see Table VIII). It should also be noted that the maximal response (E_{max}) obtained for each of the cysteine to alanine receptor mutants was equal to or greater than that obtained for hM_1-WT receptors (see Figure 12). Collectively, these data suggest that all of the receptor mutants are signaling and that differences in the phosphoinositide response would not result in decreased hM_1 receptor internalization. In fact, one might expect the opposite since the magnitude of the phosphoinositide response elicited to carbachol was greater for hM_1/2CC and hM_1/4CC; both of which did not internalize. Therefore, all of the

cysteine to alanine mutations affecting carbachol-induced internalization is not a consequence of preventing receptor signaling.

4.5 Summary

Muscarinic acetylcholine receptors have been identified as GPCRs, a large family of seven transmembrane spanning receptors that couple to a variety of effectors to induce numerous intracellular signals. Similar in structure, the muscarinic receptor subtypes (M_1-M_5) are composed of an extracellular amino-terminus, an intracellular carboxyl-terminus, and alternating intracellular (1, 2, & 3) and extracellular (1, 2, & 3) loops. M_1, M_3, and M_5 receptors couple to $G_{q/11}$ proteins to metabolize phosphoinisitides whereas M_2 and M_4 receptors couple to $G_{i/o}$ proteins to inhibit adenylate cyclase activity.

Previous observations made by our laboratory utilizing the yeast-two-hybrid system showed that an 8 amino acid domain (254-TPPGRCCR-261) in the human muscarinic receptor (hM_1) i3 loop mediates an interaction with itself. Various pharmacological assays were preformed to determine the functional role of this domain. It was concluded that a mutant hM_1 receptor lacking the domain 254-TPPGRCCR-261 internalized differently than wild-type hM_1 receptors when exposed to the muscarinic receptor-selective agonist carbachol, whereas affinity of [^{3}H]NMS binding and the potency of phosphoinositide hydrolysis elicited to carbachol were similar.

The emphasis of this research was to further characterize this small domain in the third intracellular loop of the hM_1 receptor. Using site-directed

mutagenesis, several mutant hM_1 receptors were made possessing different point mutations within the domain 252-PETPPGRCCRCCR-264. Wild-type and mutant hM_1 receptors were transiently expressed in CHO cells and the effect of each point mutation(s) on ligand affinity, agonist potency, and agonist-induced internalization was determined. The equilibrium dissociation constant (K_D) determined from intact cell [^{3}H]NMS binding assays was similar for all mutant hM_1 receptors (pK_D = ~ 9.1) tested and was comparable to that obtained for wild type receptors (pKD = 9.57 $\pm$ 0.07). Similarly, the concentration of carbachol eliciting half-maximal phosphoinositide hydrolysis (EC_{50}) was comparable between mutant (pEC_{50} = ~5.5) and wild type (pEC_{50} = 5.34 $\pm$ 0.10) hM_1 receptors. Overall, these binding and potency data indicate that none of the point mutations made prevented ligand binding or signaling of hM_1 receptors, respectively. In contrast to the binding and functional data, carbachol-induced internalization of mutant hM_1 receptors possessing either 259-CysCys/AlaAla, 262-CysCys/AlaAla, or 263 Cys/Ala point mutations was significantly reduced when compared to that of wild type receptors. These observations strongly suggest that cysteine pairs 259-260 and 262-263 play a role in the agonist-dependent internalization of hM_1 receptors, perhaps by mediating receptor dimerization/oligomerization.

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VITA

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Master of Science

Thesis: CHARACTERIZATION OF A SMALL DOMAIN IN THE I3 LOOP OF THE HUMAN MUSCARINIC M_1 RECEPTOR

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Title of Study: CHARACTERIZATION OF A SMALL DOMAIN IN THE I3 LOOP OF THE HUMAN MUSCARINIC M_1 RECEPTOR

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Scope and Method of Study: Muscarinic M_1 acetylcholine receptors are the most abundant subtype of muscarinic receptor expressed in the CNS and they are considered to play important roles in memory and cognition. When expressed in heterologous cell lines, M_1 receptors internalize when exposed to agonists, but the mechanism by which it occurs is poorly understood and may have important therapeutic implications. This study was designed to characterize the small domain 252-265 previously found to affect internalization of the hM_1 receptor in transiently expressing CHO-K1 cells. Various mutations were made inside the small domain in order to characterize the amino acids that affect internalization. To determine the degree of mutant hM_1 receptor internalization in response to agonist, the membrane impermeable muscarinic receptor selective radioligand [^{3}H]NMS was used to determine the amount of receptor plasma membrane expression before and after incubation with agonist carbachol. To determine whether mutant hM_1 receptors could still elicit phosphatidylinositol metabolism when exposed to agonist, transiently transfected CHO-K1 cells expressing the various hM_1 receptor mutants were used in phosphoinositide hydrolysis assays. To determine whether the various amino acid substitutions we made affected the affinity of the receptor for ligands or the plasma membrane expression of the receptor, radioligand binding assays were conducted.

Findings and Conclusions: Internalization is affected by mutations in the small domain 252-265 of the i3 loop of the hM_1 receptor. Studies conclude that the cysteines located at 262 and 263 are very important in the internalization rate of hM_1 receptors. The agonist-dependent internalization was affected by the mutations but the affinity of the receptor for the ligand and the function of the receptor are not affected.

ADVISOR'S APPROVAL: _____